University of Alberta

Dissecting Arabidopsis root responses to salt stress

by

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Abstract

Salt stress is one of the dominant abiotic stresses that influence plant growth and decrease the harvestable yield of all crop species. Roots are the primary site of salinity perception. To better understand salt stress responses in *Arabidopsis* roots, both the transcriptome and proteome changes in *Arabidopsis* roots treated by 150 mM NaCl for short and long terms were monitored using a full-genome oligonucleotide microarray and two-dimensional gel electrophoresis (2-DGE) based technologies, respectively. Statistical analysis of microarray results revealed that 5, 280 (22%) genes were salt-regulated with 2, 367 genes up-regulated and 2, 913 genes down-regulated by a cutoff of 2-fold. Many novel salt-responsive genes including transporters and transcription factors (TFs) were identified. A comparative proteomic analysis of salt-stressed Arabidopsis roots using 2-DGE and liquid-chromatography-tandem mass spectrometry (LC-MS/MS) allowed identification of 86 differentially expressed proteins, including many previously characterized stress-responsive proteins and proteins implicated in reactive oxygen species (ROS) scavenging, signal transduction, translation, cell wall biosynthesis as well as energy, amino acid, and hormone metabolisms. These data confirmed the modest correlation between mRNA and protein levels. Three novel salt-inducible transcription factor genes, *bHLH92*, *WRKY25* and *WRKY33* were chosen for further characterization through a combination of reverse genetic strategies including knock-out and overexpression. Quantitative RT-PCR demonstrated that the three TFs were responsive to multiple abiotic stresses. Promoter-*GUS* fusion assays revealed the spatial and developmental expression patterns and demonstrated that promoters are inducible by some abiotic stresses. Knock-out mutants for the three TFs were

identified, and stress tolerance assays showed that null mutants displayed minor differences from wild-type plants under some specific abiotic stresses, suggesting a functional redundancy exists between members of the same gene families. Constitutive overexpression of the three TFs in Arabidopsis enhanced its tolerance to salt and/or osmotic stress. Finally microarrays were employed to identify the target genes of the three TFs under saline condition, and many target genes were found to be differentially expressed between mutants and wild-type. This study not only provides a more comprehensive description of salt-responsive genes and proteins, but also contributes to elucidating the roles of bHLH and WRKY TFs in abiotic stress.

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List of Abbreviations

2-DGE, two-dimensional gel electrophoresis

4CL, 4-coumarate:CoA ligase

ABA, abscisic acid

ABC, ATP-binding cassette

ABF, ABA-responsive element binding factor

ABI, ABA insensitive

ABRC, Arabidopsis Biological Resource Center

ABRE, ABA-responsive element

ACS1, Arabidopsis cysteine synthase

AGI, Arabidopsis genome initiative

AKT, Arabidopsis K^+ transporter

APX, ascorbate peroxidase

AREB, ABRE-binding protein

ARF, auxin Response Factor

At, *Arabidopsis thaliana*

AUX/IAA, auxin/indole-3-acetic acid.

BADH, betaine aldehyde dehydrogenase

bHLH, basic helix-loop-helix

bp, base pairs

BSA, bovine serum albumin

bZIP, basic leucine zipper

CaMV, cauliflower mosaic virus

CAX, Ca^{2+}/H^+ antiporter

CBF, C-repeat-binding factor

CBL, calcineurin B-like protein

CDPK, Ca^{2+}-dependent protein kinase

CIPK, CBL-interacting protein kinase

CDS, coding region

CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate;

COMT, caffeic acid O-methyltransferase

COR, cold regulated

CPK, calcium responsive kinases

CRT, C-repeat

DAS, days after stratification

DAF, days after flowering

DD, differential display

DMSO, dimethyl sulfoxide

dNTP, deoxyribonucleotides

DRE, dehydration-responsive element

DREB, DRE-binding protein

DSP, dual specific phosphatases

DTT, dithiothreitol

EDTA, ethylenediamine tetraacetic acid

ERD, early responsive to dehydration

ERF, ethylene-responsive element binding factor

EREBP, ethylene response element binding protein

ET, ethylene

FDR, false discovery rate

Fwd, forward

G6PDH, glucose-6-phosphate-1-dehydrogenase

GA, gibberellin

GCR, G-protein-coupled receptor

GFP, green fluorescent protein;

GH, glycoside hydrolase

GPX, glutathione peroxidase

GRP, glycine-rich protein

GSH, glutathione

GST, glutathione S-transferase

GT, glycosyltransferase

GUS, β-glucuronidase

HKT, high affinity K^+ transporter

HPLC, high performance liquid chromatograph

HPTII, hygromycin B phosphotransferase

HSF, heat shock factor

ICAT, isotope-coded affinity tag

ICE1, inducer of CBF expression 1

IEF, isoelectric focusing

IPG, immobilized pH gradient

IPTG, isopropyl-beta-D-thiogalactopyranoside

JA, jasmonic acid

KIN, cold inducible

LB, left border

LEA, late-embryogenesis-abundant

LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry;

LOB, lateral organ boundary

LOS, low expression of osmotically responsive gene

LRR, leucine-rich repeat

LTP, lipid transfer proteins

MAPK, mitogen-activated protein kinase

MAPKK, mitogen-activated protein kinase kinase

MAPKKK/MAP3K, mitogen-activated protein kinase kinase kinase

MATE, multidrug and toxic compound extrusion

MDAR, Monodehydroascorbate reductase

MeJA, methyl jasmonic acid

MKS1, MAP kinase substrate 1

MLO, mildew resistance locus O

MS, Murashige-Skoog

MudPIT, multidimensional protein identification technology

MV, methyl viologen (Paraquat)

MW, molecular weight

NHX, Na^+/H^+ antiporter

NLS, nuclear localization signal

NOS, nopaline synthase

NPK, *Nicotiana* protein kinase

NPR1, nonexpressor of PR genes 1

NSCC, non-selective cation channels

Os, *Oryza sativa*

P5CR, delta(1)-pyrroline-5-carboxylate reductase

P5CS, delta(1)-pyrroline-5-carboxylate synthetase

PDH, pea DNA helicase

PERK, proline-rich extensin-like kinases

PP2C, protein phosphatase 2C

PPI, peptidylprolyl isomerase

PPP, pentose phosphate pathway

PR, pathogenesis-related

PRX/PER, peroxidase

pI, Isoelectric point

PTM, post-translational modification

qRT-PCR, quantitative RT-PCR

QTL, quantitative trait locus

RAB, responsive to ABA

RB, right border

RCD1, radical-induced cell death 1

RD, response to dehydration

REL, relative electrolyte leakage

RING, really interesting new gene

RLCK, receptor like cytoplasmic kinase

RLK, receptor-like kinases

ROS, reactive oxygen species

RPS6K, ribosomal protein S6 kinases

RT-PCR, reverse transcription-polymerase chain reaction

Rvs, reverse

SA, salycilic acid

SAGE, serial analysis of gene expression

SAM, significance analysis of microarray

SCOF1, soybean C2H2-type zinc finger protein1

S.D., standard deviation

SDS, sodium dodecyl sulfate

SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SFR, sensitive to freezing

SGBF1, soybean G-box binding bZIP transcription factor 1

SOD, superoxide dismutase

SOS, salt overly sensitive

SSH, suppressive subtractive hybridization

STEM, short time-series expression miner

TAIR, the Arabidopsis Information Resource

TAP, tandem affinity purification

TCA, tricarboxylic or trichloroacetic acid

TF, transcription factor

TIGR, the Institute of Genome Research

TL, translational leader

TSAP, thermo-sensitive alkyline phosphatase

UBQ, ubiquitin

UTR, untranslated region

WAK, wall-associated kinase

WT, wild-type

X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

XTH, xyloglucan endotransglucosylase

ZIM, Zinc-finger protein expressed in inflorescence meristem

Chapter 1: Literature Review

Introduction

Salinity is a severe and increasing constraint on the productivity and yield of agricultural crops and threatens food sustainability worldwide. Saline soil has toxic levels of NaCl and Na_2SO_4 and by definition, the electrical conductivity (EC) of saturation extracts of saline soil exceeds more than 4.0 dS/m (deciSiemen/metre, 1.0 dS/m ≈ 10mM NaCl). However, many crops are also affected by soil with an EC less than 4 dS/m (Chinnusamy *et al.*, 2005).

It is estimated that 45 Mha of the current 230 Mha of irrigated land are salt-affected (20%) (Munns 2005). Primary salinity in soil mostly originates from previous exposure to sea water. Secondary salinization of arable land arises from low-quality irrigation water and improper drainage, which has led to the decline of arable land, and it is threatening the long-term sustainability of many present large-scale irrigation systems. Excess salts in the soil interfere with mineral nutrition and water uptake, and lead to undue accumulation of toxic ions in plants. Salinity imposes two stresses on plant tissue: an initial water-deficit that results from the relatively high solute concentration in the soil, which is caused by the osmotic effect of salt around the roots, and the subsequent ion-specific stress resulting from altered K^+/Na^+ ratio. Although sodium is an essential micronutrient for some C_4 plants, in which Na^+/pyruvate co-transporters import pyruvate into mesophyll chloroplasts, most crop plants are natrophobic (Ohnishi *et al.*, 1990). With regards to salt sensitivity, plants can be divided into two groups: glycophytes, which are salt sensitive, and halophytes, which are salt tolerant that can grow in 200 to 500 mM NaCl. Halophytes can be further divided into two subcategories, euhalophytes or facultative halophytes, which require salt for optimum growth (e.g. *Suaeda maritima, Atriplex nummularia),* and miohalophytes, which are capable of growth under high levels of salt, although the growth rate is inhibited by salt, e.g. *Atriplex hastata, Spartina townsendii* and sugar beet (*Beta vulgaris* L.) (Glenn *et al.*, 1999). Nearly all salt tolerant plants are angiosperms, and these species are widely distributed amongst angiosperms, indicating a polyphyletic origin or that halophytes are primitive genetic remnants of different families (Flowers 2004).

Considerable efforts were taken in the past several decades to understand the underlying genetic bases of the physiology of salinity tolerance, but little progress was made until the introduction of Arabidopsis as a genetic model to study salinity tolerance (Zhu 2000), partly due to the difficulty of conducting genetic analysis in most of the well-characterized halophytes. As Arabidopsis is a glycophyte that does not exhibit salinity tolerance even approaching that of halophytes, its utilization in salinity tolerance studies was initially thought to be inappropriate. However, forward genetic studies using Arabidopsis have yielded essential knowledge about the genetic bases of salinity tolerance (Zhu 2002). In the following sections, I will review and discuss the toxicity of salt on plants, cellular dehydration caused by salinity, the molecular basis of ion homeostasis, the role of abscisic acid (ABA) in salt stress signaling, the cross-talk and specificity between abiotic stresses, as well as the utilization of various genes for engineering salt-tolerant plants.

Effects of salt toxicity on plant physiology

Most commonly, high Na^+ and Cl^- in the soil and water cause salt stress. Salt stress has three effects; i.e. it reduces water potential (osmotic stress) and causes ion imbalance or disturbances in ion homeostasis and at the same time leads to cellular toxicity (Hasegawa *et al.*, 2000). General symptoms of damage caused by salt stress are growth inhibition due to dehydration as a result of altered water status, accelerated development, senescence and death during prolonged exposure (Munns 2005). The immediate response to salt stress is a reduction in the rate of leaf surface expansion leading to cessation of expansion as salt concentration increases (Wang and Nil, 2000). Growth inhibition is the primary injury that leads to other symptoms although programmed cell death (PCD) may also occur under severe salinity shock. Since salt stress involves both osmotic and ionic stress (Munns 2002), growth suppression is directly related to the total concentration of soluble salts or osmotic potential of soil water. Water potential and osmotic potential of plants become more negative with an increase in salinity (Hernandez *et al.*, 1999; Romeroaranda *et al.*, 2001). Salt stress induces the synthesis of ABA (Jia *et al.*, 2001), which closes stomata when transported to guard cells (Zeevaart and Creelman, 1988). As a result of stomatal closure, photosynthesis declines

and photoinhibition and oxidative stress occur. Another reason for decreased photosynthetic rate is that chlorophyll and total carotenoid contents of leaves decrease in general under salt stress (Hernandez *et al.*, 1999). Oxidative stress occurs under salt stress because of the formation of reactive oxygen species (ROS) such as superoxides and hydroxy and peroxy radicals. The ROSs that are by-products of hyperosmotic and ionic stresses cause membrane dysfunction and cell death (Bohnert and Jensen, 1996).

Water deficit due to salinity stress

A major initial consequence of NaCl stress is a reduced ability to take up water from the soil, which leads to slower growth. The amount of water transport depends on the motive force generated by an osmotic gradient (difference in water potential between two compartments) and water permeability. When extracellular water potential is reduced by salinity, the osmotic gradient can be restored by decreasing the intracellular water potential with the accumulation of compatible solutes, also known as osmolytes or osmoprotectants (Chen and Murata, 2000). These solutes include proline, glycine betaine, raffinose, mannitol, sorbitol, sucrose and trehalose, which are all relatively small, non-toxic compounds that can stabilize proteins and cellular structures, and are proposed to increase the osmotic pressure of plant cells. Many genes involved in osmoprotectant biosynthesis are upregulated under salt and drought stresses (Chen and Murata, 2000). Over-expression of genes involved in the biosynthesis of compatible osmolytes, either from *E. coli* or plants, in transgenic *Arabidopsis*, rice, wheat, tobacco and *Brassica* shows enhanced germination, growth, survival, recovery, and production under salt and osmotic stresses (Bohnert and Jensen, 1996; Chinnusamy *et al.*, 2005). Cell-to-cell water transport is facilitated by membrane proteins called aquaporins, which form water channels (Maurel and Chrispeels, 2001). Some aquaporin genes are regulated by salt or drought stress. Over-expression of a barley aquaporin increased salt sensitivity in transgenic rice plants, indicating that the increase in water conductance of leaf cells accelerates water loss from transgenic leaves (Katsuhara *et al.*, 2003).

Another group of water stress induced proteins includes late-embryogenesis-abundant proteins (LEAs) and their close relatives, dehydrins. These proteins may help to stabilize protein structures when cells start to lose water. Xu *et al.* (1996) reported that

the expression of a barley LEA protein, HVA1, confers tolerance to water deficit and salt stress in transgenic rice. However, overexpression of a single LEA-type protein is not always sufficient to confer plant stress tolerance, suggesting that LEA-type proteins might function synergistically with other proteins (Wang *et al.*, 2003).

Sodium entry and efflux of plant cells

While Na^+ is deleterious for plant growth, K^+ is an essential element and is required by plants in large quantities. Movement of salt into roots and to shoots is driven by transpirational flux required to maintain the water status of plants. Na^+ entry into the cells of the root is passive and uniporter or ion channel type transporters are the candidates for sodium influx (Tester and Davenport, 2003). Many K^+ transport systems, such as Na^+/K^+ symporters, have some affinity for Na^+, which can enter cells through the high-affinity K^+ transporter HKT1 and non-selective cation channels (NSCCs). AtHKT1 is a low affinity Na^+ transporter and mediates Na^+ entry into the root cells of *Arabidopsis* under salt stress, and mutation in *HKT1* suppresses Na^+ accumulation and hypersensitivity of the plants to Na^+ (Berthomieu *et al*, 2003; Rus *et al*, 2001). NSCCs catalyze passive fluxes of cations through plant membranes and do not show much selectivity between monovalent cations; several NSCCs are also permeable to divalent cations. NSCCs are directly involved in a multitude of stress responses, growth and development, uptake of nutrients and calcium signaling (Demidchik and Maathuis, 2007). Accumulated evidence has supported that NSCCs are the main pathway for Na^+ entry into the roots, at high soil NaCl concentrations (Tester and Davenport, 2003). In wheat, a low affinity cation transporter (LCT1) was isolated using a screen for complementation of K^+ uptake mutant of yeast (Schachtman *et al.*, 1997). In yeast, LCT1 catalyzed uptake of many cations, including Na^+, Rb^+, and Ca^{2+}. Introduction of wheat LCT1 into yeast causes hypersensitivity to sodium in yeast, which was mitigated by the addition of high (20 mM), but not low (2 mM) Ca^{2+} to the medium (Amtmann *et al.*, 2001). It was therefore suggested that LCT1 may be a component of the Ca^{2+}-insensitive Na^+ influx seen in wheat roots, as it was not saturated at low external Na^+ (7 mM), a property attributed to NSCCs (Amtmann *et al.*, 2001). Similar transporers in rice or

Arabidopsis have not been identified, suggesting LCT1 may be unique to wheat (Aspe and Blumwald, 2007).

Sodium extrusion from plant cells is mediated by the plasma membrane Na^+/H^+ antiporter SOS (Salt Overly Sensitive)1, which is composed of 12 trans-membrane domains (Shi *et al.*, 2000). Activation of SOS1 is controlled by SOS3 and SOS2. The *SOS3* gene encodes a myristoylated Ca^{2+} binding protein, also known as CBL4 (calcineurin B-like protein 4). In response to Ca^{2+} perturbation, SOS3 changes its conformation and transduces a signal downstream by interacting with an effector kinase, SOS2, also known as CIPK24 (CBL-interacting protein kinase 24). *SOS2* encodes a novel serine/threonine protein kinase with an N-terminal catalytic and C-terminal regulatory domain. SOS3 interacts with SOS2 via a FISL motif and relieves the protein from autoinhibition, thereby making the kinase active (Fig. 1-1, Zhu, 2002). Na^+ extrusion is powered by a plasma membrane H^+-ATPase that generates an electrochemical H^+ gradient and allows Na^+/H^+ antiporters to couple the passive movement of H^+ inside the cells with the active extrusion of Na^+. Overexpression of *SOS1* in *Arabidopsis* shows improved salt tolerance, demonstrating that limiting Na^+ accumulation in cytoplasm is an effective way to attain salt tolerance (Shi *et al.*, 2003). The discovery of SOS genes paved the way for elucidation of a novel pathway linking the Ca^{2+} signaling to salt stress (Zhu, 2002 & 2003).

Sodium compartmentation

Compartmentation of Na^+ in the vacuole allows plants to maintain a lower Na^+ concentration at the sites of biochemical reactions in the cytosol, and yet maintain a lower overall osmotic potential. Influx of Na^+ into the vacuole occurs through Na^+/H^+ antiporters, which use the proton gradient generated by V-type H^+-ATPase and H^+-pyrophosphatase (PPase) (Apse *et al.* 1999). Salinity up-regulates the expression of a V-type H^+-ATPase gene (Golldack and Dietz 2001) and a vacuolar Na^+/H^+ antiporter gene (Gaxiola *et al.* 1999; Shi and Zhu 2002).

In *Arabidopsis*, the *AtNHX1* gene encodes a tonoplast Na^+/H^+ antiporter. *AtNHX1* expression under salt stress is partially dependent on ABA biosynthesis and ABA signaling through the protein phosphatase 2C-ABI1 (ABA insensitive 1). Salt-stress

induced up-regulation of AtNHX1 expression is lower in ABA deficient mutants (*aba2-1* and *aba3-1*) and in the ABA insensitive mutant, *abi1-1* (Shi and Zhu, 2002). It was also shown that SOS2 regulates the AtNHX1 activity (Qiu *et al.*, 2004). More recently, in a yeast two-hybrid analysis, it was shown that SOS2 interacts directly with V-ATPase regulatory subunits B1 and B2, suggesting that regulation of V-ATPase activity is an additional key function of SOS2 in coordinating changes in ion transport during salt stress and in promoting salt tolerance (Batelli *et al.*, 2007). Overexpression of AtNHX1 in *Arabidopsis*, canola and tomato resulted in significantly higher salt tolerance (Apse *et al.*, 1999; Zhang *et al.*, 2001; Zhang and Blumwald, 2001). Overexpression of AVP1 in Arabidopsis plants, a vacuolar H^+-PPase, showed enhanced salt tolerance as well as drought tolerance (Gaxiola *et al.*, 2001). These studies confirm that sequestration of Na^+ into the vacuole is an important trait of salt tolerance in plants (Fig. 1-1).

Reactive oxygen species and antioxidants

A secondary aspect of salinity stress in plants is the increased abundance of reactive oxygen species (ROS). In plants, ROSs, including superoxide radicals, hydrogen peroxide and hydroxyl radicals, are continuously produced predominantly in chloroplasts, mitochondria, and peroxisomes (Apel and Hirt, 2004). In other circumstances, plants appear to purposefully generate ROS as signaling molecules to control various processes including pathogen defense, programmed cell death, and stomatal behavior (Apel and Hirt, 2004). However, ROSs can also cause oxidative damage to membrane lipids, proteins and nucleic acids (Smirnoff, 1993). Therefore, production and removal of ROS must be strictly controlled. However, the equilibrium between production and scavenging of ROS may be perturbed by a number of adverse abiotic stress factors such as high salinity, high light, drought, low temperature, high temperature, and mechanical stress (Mittler *et al.*, 2004).

Studies on the role of ROS in signaling in plants revealed that ROS might be sensed by two-component histidine kinases (HKs), and activate mitogen-activated protein kinase (MAPK) signaling pathways (Jonak *et al.*, 2002). ROS might change gene expression by targeting and modifying the activity of transcription factors. In

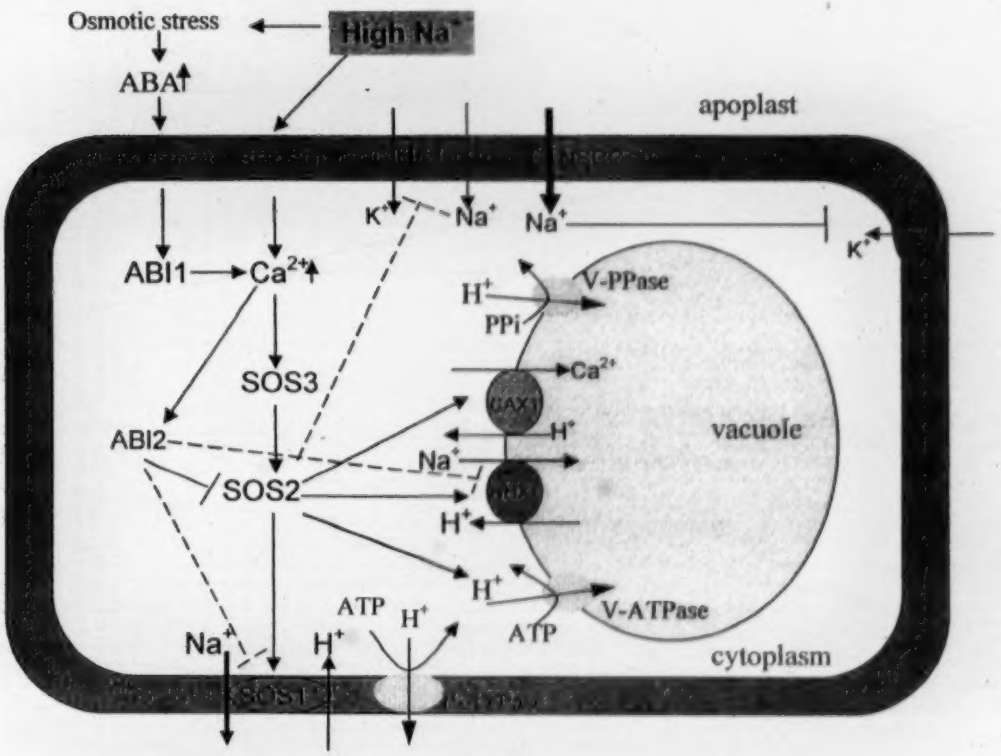

Fig. 1-1. Regulation of sodium, potassium and calcium homeostasis in plant cells. Higher concentration of extracellular NaCl is perceived by an unknown receptor at the plasma membrane (PM), which induces a cytosolic calcium perturbation. SOS3 senses the Ca^{2+} increase and interacts with SOS2. This interaction relieves SOS2 of its auto-inhibition and activates it, which forms a complex with SOS3 to phosphorylate PM-localized Na^+/H^+ antiporter SOS1, resulting in efflux of excess Na^+ ions. SOS3–SOS2 complex inhibits the low affinity Na^+ transporter HKT1 activity thus restricting Na^+ entry into the cytosol. SOS2 interacts and activates the vacuolar Na^+/H^+ exchanger NHX1 resulting in sequestration of excess Na^+ ions into the vacuole. SOS2 also activates the H^+/Ca^{2+} antiporter CAX1 on the vacuolar membrane reinstating cytosolic Ca^{2+} homeostasis. High salinity also causes osmotic stress, which induces the synthesis of ABA. A protein phosphatase 2C (ABI1) can regulate NHX1 expression. The protein phosphatase ABI2 physically interacts with SOS2 and inactivates SOS2. High intracellular Na^+ also inhibits the K^+ transporter-AKT1 on PM. Dotted lines indicate possible regulations (Adapted from Zhu 2003 and Chinnusamy *et al.*, 2005).

Arabidopsis, H_2O_2 activates the MAPKs, MPK3 and MPK6 via MAPKKK ANP1, and overexpression of a tobacco ANP1 orthologue, NPK1, in transgenic tobacco plants

resulted in increased tolerance to heat shock, freezing, and salt stress (Kovtun *et al.*, 2000). H_2O_2 also increases expression of the *Arabidopsis* nucleotide diphosphate kinase (AtNDPK) 2 (Moon *et al.*, 2003). Overexpression of AtNDPK2 reduced accumulation of H_2O_2 and enhanced tolerance to multiple stresses including cold, salt, and oxidative stress. The effect of NDPK2 might be mediated by the MAPKs, MPK3, and MPK6 because NDPK2 can interact and activate the MAPKs (Moon *et al.*, 2003).

Plants use two different mechanisms for ROS scavenging. Nonenzymatic scavenging of ROS uses cellular ascorbic acid, glutathione (GSH), tocopherol, flavonoids, alkaloids, and carotenoids. Enzymatic scavenging employs detoxifying enzymes including superoxide dismutases (SODs), catalases (CATs), ascorbate peroxidases (APXs), and glutathione peroxidases (GPXs) (Apel and Hirt, 2004). SODs act as the first line of defense against ROS, dismutating superoxide to H_2O_2. APX, GPX, and CAT subsequently detoxify H_2O_2. ROS detoxification could provide enhanced plant resistance to salt and other abiotic stresses. Transgenic plants overexpressing some of these enzymes demonstrated increased tolerance to osmotic, temperature and oxidative stresses (Alscher *et al.*, 2002; Roxas *et al.*, 1997; Wang *et al.*, 1999).

A recent report indicated that the plasma membrane Na^+/H^+ antiporter SOS1 is also involved in oxidative stress tolerance (Katiyar-Agarwal *et al.*, 2006). It was found that under salt or oxidative stress, SOS1 interacts through its predicted cytoplasmic tail with RCD1 (radical-induced cell death), a regulator of oxidative-stress responses. Without stress treatment, RCD1 is localized in the nucleus. Under high salt or oxidative stress, RCD1 is found not only in the nucleus but also in the cytoplasm. Like *rcd1* mutants, *sos1* mutant plants show an altered sensitivity to oxidative stresses. The *rcd1* mutation causes a decrease in salt tolerance and enhances the salt-stress sensitivity of *sos1* mutant plants. Several genes related to oxidative-stress tolerance were found to be regulated by both RCD1 and SOS1. These results shed light on the cross-talk between the ion-homeostasis and oxidative-stress detoxification pathways involved in plant salt tolerance.

The role of ABA in salt stress

ABA plays an important role in many aspects of plant growth and development and

also in abiotic stress response. In fact, the role of ABA in response to abiotic stresses has long been recognized and extensively studied utilizing mutants deficient in ABA synthesis and sensitivity (Finkelstein *et al.*, 2002; Nambara and Marion-Poll, 2005). Salt, drought, and to a lesser extent, cold stresses, increase the biosynthesis and accumulation of ABA, which then is rapidly catabolised after stress relief (Koornneef *et al.*, 1998; Cutler and Krochko, 1999; Schwartz *et al.*, 2003). Salt stress induced ABA accumulation appears to be due to both ionic and osmotic stresses in roots, while that in the leaf is mainly due to osmotic stress (Jia *et al.* 2002).

The plethora of ABA responses is initiated by ABA perception. Three different ABA receptors, the nuclear RNA binding protein flowering time control protein (FCA), the chloroplast protein Mg chelatase H subunit, and the plasma membrane-localized GCR2 (G-protein-coupled receptor 2) have been identified (Razem *et al.*, 2006; Shen *et al.*, 2006; Liu *et al.*, 2007); however, a more recent report points out that GCR2 is not an ABA receptor in seed germination and early seedling development. Instead, GCR2 is most likely a plant homolog of bacterial lanthionine synthetases (Gao *et al.*, 2007). Genetic disruption of the single Gα prototype of heterotrimeric G protein GPA1 present in *Arabidopsis* caused insensitivity of stomatal opening to inhibition by ABA (Wang *et al.*, 2001). GPA1 presumably represents a downstream effector of GCR1, a putative G protein-coupled receptor which acts as a negative regulator of GPA1-mediated ABA responses in guard cells (Pandey and Assmann, 2004). Evidence suggests that G-proteins modulate ABA responses (Wang *et al.*, 2001; Lemichez *et al.*, 2001; Zheng *et al.*, 2002). The Rho-like small G-protein, ROP10, negatively regulates ABA-mediated stomatal closure, germination, and growth inhibition (Zheng *et al.*, 2002). Recruitment of ROP10 to the plasma membrane required a functional farnesylation site, which supports the observed role of a farnesyltranferase β subunit ERA1 as a negative regulator of ABA responses upstream of cytosolic Ca^{2+} (Allen *et al.*, 2002). ROP2 and ROP6/AtRac1 work as pleiotropic negative modulators of ABA responses linked to actin skeleton reorganization and vesicle transport and required for stomatal closure or tip growth (Lemichez *et al.*, 2001; Jones *et al.*, 2002). In line with this conclusion, impairment of vesicle transport or fusion by deficiency in the syntaxin OSM1 (Osmotic Stress–sensitive Mutant 1), resulted in ABA-insensitive stomatal regulation (Zhu *et al.*,

2002). Thus, OSM1 plays a critical role in root growth and in ABA regulation of stomatal responses under osmotic stresses (Zhu *et al.*, 2002).

ABA signaling for stomatal closure and gene expression is transduced through Ca^{2+} (Schroeder *et al.*, 2001). A growing number of protein kinases, such as calcium-dependent protein kinases (CDPKs) as well as protein phosphatases such as PP2Cs, have been implicated in ABA-induced stomatal closure and gene expression (Christmann *et al.*, 2006). ABA transcriptionally upregulates a number of PP2Cs in *Arabidopsis* which act as negative regulators of ABA responses (Saez *et al.*, 2004; Kuhn *et al.*, 2006). Protein phosphatases ABI1 and the highly homologous ABI2 are partly redundant key regulators of ABA-invoked seed dormancy, stomatal closure, and growth inhibition (Finkelstein and Rock, 2002). Previous studies showed that salt-stress induced ABA accumulation regulates the SOS pathway through the ABI2 protein phosphatase 2C (Ohta *et al.*, 2003). ABI2 interacts with the protein phosphatase interaction (PPI) motif of SOS2. This interaction is abolished by the *abi2-1* mutation, which enhances tolerance of seedlings to 150 mM NaCl and causes ABA insensitivity (Ohta *et al.*, 2003). Therefore, ABI2 may negatively regulate salt tolerance either by inactivating SOS2, or the SOS2 regulated Na^+/H^+ antiporters such as SOS1 or NHX1 (Fig. 1-1).

Osmotic stress imposed by high salt or drought is transmitted through at least two pathways: ABA-dependent and ABA-independent. ABA induced expression often relies on the presence of a *cis* acting element called ABA-responsive element (ABRE) (Uno *et al.*, 2000). Increased ABA level under stress conditions up-regulates many LEA-like stress responsive genes, including *RD* (responsive to dehydration), *ERD* (early responsive to dehydration), *KIN* (cold inducible), and *RAB* (responsive to ABA). Two Arabidopsis ABA-deficit mutants, *low expression of osmotically responsive gene mutant5 (los5)* and *los6* are impaired in the expression of stress responsive genes, such as *RD29A, COR15A, COR47, RD22*, and *P5CS*, under salt and osmotic stresses and they are also defective in ABA biosynthesis. *LOS5* encodes a molybdenum cofactor sulfurase (MCSU) and is allelic to *ABA3*, while *LOS6* allelic to *ABA1*, encodes a zeaxanthin epoxidase (ZEP) (Xiong *et al.*, 2001, 2002a). These results demonstrate that stress responsive gene expression under salinity is mediated by ABA. Genetic analysis

indicates that there is no clear line of demarcation between ABA-dependent and ABA-independent pathways and the components involved may often cross talk or even converge in the signaling pathway (Chinnusamy *et al.*, 2004; Seki *et al.*, 2003; Xiong *et al.*, 2002b; Zhu, 2002). Calcium, which serves as a second messenger for various stresses, represents a strong candidate, which can mediate such cross-talk (Sanders *et al.*, 2002).

Salt stress signaling

Salt stress affects cellular ion homeostasis as well as osmotic homeostasis. Hence, excess ions (Na^+ and Cl^-) and osmotic stress-induced turgor change may act as inputs for salt stress signaling. Na^+ entry into cells causes membrane depolarization, which is known to activate Ca^{2+} channels (Zhu, 2002). Salinity induces the biosynthesis and accumulation of ABA and also induces accumulation of ROSs. Current evidence suggests that the primary salt stress signals (ionic and osmotic stress) are transduced through Ca^{2+} as well as receptor kinase pathways, while the secondary salt stress signals such as ABA and H_2O_2 also regulate plant salt tolerance (Chinnusamy *et al.*, 2005).

High salinity results in increased cytosolic Ca^{2+} that is transported from the apoplast as well as the intracellular compartments (Knight *et al.*, 1997). The ionic aspect of salt stress is signaled through the SOS pathway where a calcium-responsive SOS3-SOS2 protein kinase complex controls the expression and activity of ion transporters such as SOS1 (Liu and Zhu, 1998). SOS2 kinase also positively regulates the activities of AtNHX1 and a vacuolar Ca^{2+}/H^+ exchanger CAX1 and may negatively regulate AtHKT1 (Fig. 1-1, Zhu, 2003). More recently, genetic and biochemical analyses demonstrated that the calcium sensor CBL10 protein physically interacts with SOS2 (CIPK24), and the CBL10-SOS2 complex is associated with the vacuolar compartments that are responsible for salt storage and detoxification in plant cells (Kim *et al.*, 2007). These findings suggest that CBL10 and SOS2 constitute a novel salt-tolerance pathway that regulates the sequestration/compartmentalization of Na^+ in plant cells (Kim *et al.*, 2007). In another report, SOS2 is found to interact with subunits of the vacuolar H^+-ATPase (V-ATPase) and upregulate its transport activity (Batelli *et al.*,

2007). Therefore, coordination exists between the transporters in the tonoplast and plasma membranes in part through the SOS2 kinase (Cheng *et al.*, 2004; Qiu *et al.*, 2004). Recently, the identification and characterization of the SOS pathway in rice supports the conception that this pathway is conserved across diverse taxa (Martinez-Atienza *et al.*, 2007).

Osmotic stress activates several protein kinases including mitogen-activated protein kinases (MAPKs), which mediate osmotic homeostasis and/or detoxification responses. Plant MAPKs are implicated in signaling development, cell division, hormones, biotic, and abiotic stresses. Salt stress quickly activates MAPKs in plants. Correlative evidence suggests that the ROS mediated signaling under salt stress occurs through MAPKs (Chinnusamy *et al.*, 2004). Transcript levels for a number of protein kinases including a two component histidine kinase MAPKKK, MAPKK and MAPK increase in response to osmotic stress (Mikolajczyk *et al.*, 2000). This ultimately results in the accumulation of osmoprotectants that helps reestablish the osmotic balance, protection from stress damage or repair mechanisms by induction of LEA/dehydrin-type stress genes.

Specificity and cross talk in abiotic stresses

Plants grow in a changing environment and experience various environmental stresses. To respond and adapt to these abiotic stresses, plants have evolved mechanisms for various physiological and metabolic changes that increase their tolerance. It is increasingly clear that signaling pathways are actually part of a more complex hierarchy of signaling networks and that common components are shared between different pathways. Many abiotic stresses induce the biosynthesis and accumulation of ABA, which is known to mediate the expression of many genes in response to osmotic, drought and cold stress. However, the fact the expression of some stress responsive genes is unaffected in ABA-deficient and ABA-insensitive mutants indicates the presence of ABA-independent pathways. It is proposed that at least four signaling pathways functions under drought stress: two are ABA-dependent and two are ABA-independent (Fig. 1-2, Seki *et al.*, 2003). ABA-dependent pathways involve basic leucine-zipper and MYB/MYC type transcription factors that recognize ABRE (Uno *et*

al., 2000) and MYB/MYC recognition sequences in the downstream LEA-like genes (Abe *et al.*, 2003), respectively (Fig. 1- 2). ABA-independent signaling pathways are mediated through transcription factors C-repeat binding factors (CBFs) or dehydration responsive element binding factors (*DREB*s) that activate DRE/CRT *cis*-elements of LEA-type protein encoding genes (Stockinger *et a l.*, 1997; Liu *et al.*, 1998; Jaglo-Ottosen *et al.*, 1998).

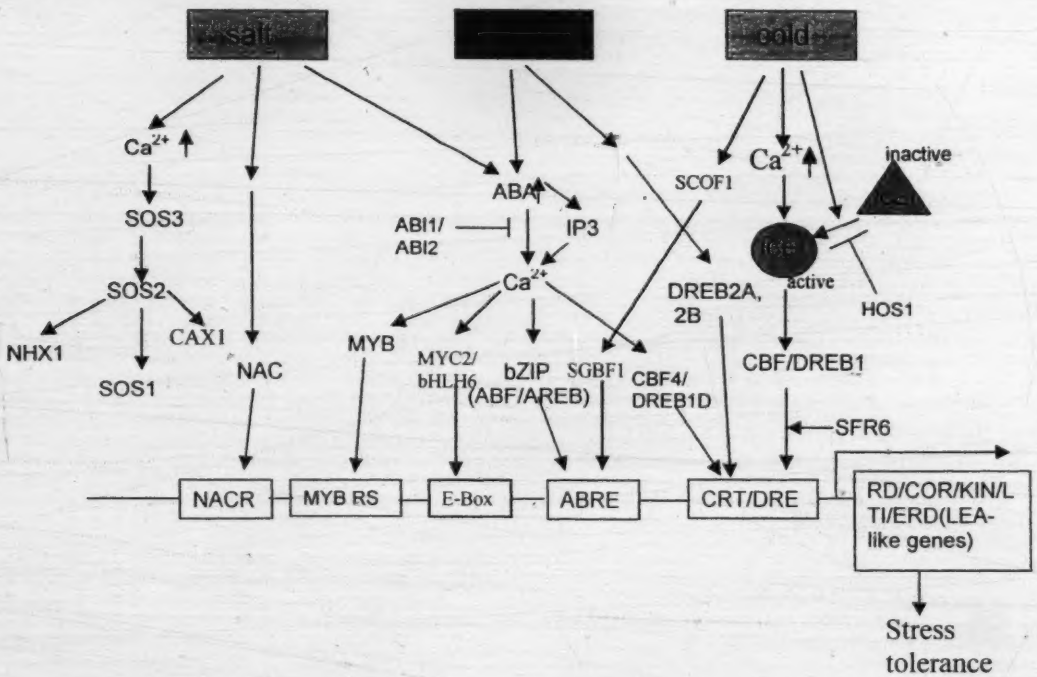

Fig. 1-2. Transcriptional cascades of salt, drought and cold signal transduction. Salinity works through SOS pathway to reinstate cellular ionic equilibrium or through transcription factors MYB/MYC, bZIP, CBF4/DREB1D, DREB2 to activate downstream *LEA*-like genes. Drought stress works through both ABA-dependent and ABA-independent pathway and shares some components with salt stress. Cold stress works through ICE1 and trigger transcription factors CBF1/DREB1B, CBF2/DREB1C and CBF3/DREB1A to mediate their influence on stress genes mainly via ABA-independent pathway. Transcription factors such as CBF/DREB1, DREB2A, DREB2B, bZIP, MYC (bHLH) and MYB interacts with CRT/DRE, ABRE, MYBRS or E-box elements in the promoter of *LEA*-like genes such as *RD29A*, *RD29B*, *COR15a, KIN1, ERD10* as well as *RD22* to induce the expression of these genes. (adapted from Xiong *et al.*, 2002b; Chinnusamy *et al.*, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006).

In Arabidopsis, the promoter regions of several cold- and drought-induced COR genes (*COR6.6*, *COR15a*, and *COR78*) contain a 9 bp consensus sequence named C-repeat (CRT)/drought responsive elements (DRE), which stimulates gene expression in response to cold, drought, and salt, but not in response to the exogenous application of ABA (Yamaguchi-Shinozaki and Shinozaki, 1993). Using the yeast one-hybrid system, five different DRE/CRT binding genes, namely, *CBF1(DREB1B)*, *CBF2(DREB1C)*, *CBF3(DREB1A)*, *DREB2A* and *DREB2B* were isolated and characterized (Stockinger *et a l.*, 1997; Gilmour *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999). The three *DREB1* genes are specifically induced by cold whereas the two *DREB2* genes are specifically induced by drought and salt stresses (Liu *et al.*, 1998; Nakashima *et al.*, 2000), and all of them are able to transcriptionally activate the expression of *COR* genes. Another CBF transcription factor, *CBF4* was later isolated from Arabidopsis (Haake *et al.*, 2002). *CBF4* gene expression is up-regulated by drought stress and ABA treatment, but not by low temperature, and it was also found that ABA biosynthesis is required for the proper drought-induced induction of *CBF4* expression (Haake *et al.*, 2002, Fig. 1-2). An example of cross talk between ABA-dependent and ABA-independent stress-responsive pathways is well documented by the transcription of the *COR* genes. As the COR gene promoters contains both DRE/CRT and ABRE consensus sequences, ABA-independent TFs (DREB1 and DREB2) and ABA-dependent basic leucine zipper (bZIP) TFs exist (Fig. 1-2, Uno *et al.*, 2000). The discovery and characterization of bZIP type ABRE binding factors (ABF or AREB) demonstrated that ABA is required for their full activations (Choi *et al.*, 2000; Uno *et al.*, 2000). More direct evidence for the existence of cross talk between abiotic stresses comes from the microarray profiling of trancriptome changes under cold, salt, drought and osmotic stresses (Kreps *et al.*, 2002; Seki *et al.*, 2002). For instance, Seki *et al.*(2002) found that there is greater cross talk between drought and high salinity than between cold and salt stress signaling processes by microarray analysis.

A MAPK cascade, which has already been discussed previously in the context of ABA- and osmotic mediated signalling is believed to be the strongest evidence for cross-talk during abiotic stress signalling in plants based on the observation that diverse signals produced by different stress pathways converge at this cascade (Chinnusamy *et*

al., 2004). MAPK cascades are vital to fundamental physiological functions involved in hormonal responses, cell cycle regulation, abiotic stress signaling, and defense mechanisms (Tena *et al.*, 2001). The Arabidopsis genome encodes approximately 60 MAPKKKs, 10 MAPKKs and 20 MAPKs (Tena *et al.*, 2001). As signals transduced to the 60 MAPKKKs have to be transduced through 10 MAPKKs to 20 MAPKs, it provides a scope for cross-talk between different stress signals. Members of MAPK cascades are activated by H_2O_2, ABA and by both abiotic (wounding, drought, salt, cold), and biotic stresses in Arabidopsis, rice and tobacco (Mizoguchi *et al.*, 1996; Ichimura *et al.*, 1998, 2000; Kovtun *et al.*, 2000; Yuasa *et al.*, 2001; Xiong and Yang 2003), which suggests that MAPK cascades act as points of convergence in stress signaling. Ichimura *et al.*(1998) identified a putative Arabidopsis MAPK cascade consisting of AtMEKK1, AtMEK1/AtMKK2, and AtMPK4, in which salt stress induces the expression and activity of AtMEKK1 (Ichimura *et al.*, 2000), which activates AtMPK4 in vitro (Huang *et al.*, 2000). The Arabidopsis MAPK phosphatase 1 (*mkp1*) mutant is resistant to salinity but hypersensitive to genotoxic stress induced by UV-C. A yeast two-hybrid screen showed that MKP1 could interact with MPK3, MPK6 and MPK4, and the interaction was strongest with MPK4 (Ulm *et al.*, 2002). Previous studies suggested that MPK3, MPK4 and MPK6 are implicated in salt stress signaling (Mizoguchi *et al.*, 1996; Ichimura *et al.*, 2000). Taken together, these suggest that MKP1 is a negative regulator of salt stress signaling through MAPK, while it functions as positive regulator in genotoxic stress tolerance (Ulm *et al.*, 2002). Microarray analysis of *mkp1* identified that the transcript level of a putative vacuolar Na^+/H^+-antiporter (At4g23700, CHX17) was increased under salt stress (Ulm *et al.*, 2002), which indicates that this gene may be upregulated by a MAPK cascade that is negatively controlled by MPK1. Transient expression studies in Arabidopsis mesophyll protoplasts showed that MPK3 and MPK4 function downstream of the flagellin receptor FLS2 (Asai *et al.*, 2002). Studies from alfalfa and tobacco also showed that a MAPK cascade is a node of cross-talk in biotic and abiotic stress signaling (Baluska *et al.*, 2000; Cardinale *et al.*, 2000). Thus biotic and abiotic stress signals converge at the level of MAPK cascades (Cardinale *et al.*, 2002; Fujita *et al.*, 2006).

Strategies of developing salt tolerant plants

Plant growth under salt stress depends on the reestablishment of proper cellular ion homeostasis with other concomitant processes such as detoxification and growth regulation. Low cytosolic Na^+ content is maintained through the concerted interplay of regulated ion uptake, vacuolar compartmentation, and active extrusion to the extracellular milieu (Zhang *et al.*, 2004). Increased salt tolerance requires identification of genes and germplasm that perform these processes.

Unlike plant resistance to biotic stresses, which is mostly dependent on monogenic traits, the responses to abiotic stresses are multigenic and thus more difficult to control and engineer (Vinocur and Altman, 2005). There are two main strategies of producing salt-tolerant plants based on genetic information: marker-based breeding through QTL mapping and transgenic approaches either by introducing novel genes or altering the expression levels of existing genes to affect the degree of salt stress tolerance. QTLs and marker-assisted selection have advantages over direct phenotypic screening. Ren *et al* (2005) reported that allelic variation in *OsHKT8*, which encodes a Na^+ transporter, contributes to enhanced capacity of a salt-tolerant variety of rice to maintain shoot K^+ homeostasis under NaCl stress. The drawbacks in using marker-assisted breeding are "linkage drag" of undesirable traits due to the large size of regions of chromosomes identified by QTLs and, the influence of environment and genetic background on the QTLs that are identified (Flowers, 2004).

Transgenic strategies for increasing salt stress tolerance rely on the expression of genes that are involved in signaling, ion homeostasis, or enzymes for the synthesis of functional and structural metabolites (Table 1-1), which are discussed in more details as below.

Ion transporters

Under salt stress condition, plants use three strategies to maintain a high K^+/Na^+ ratio in the cytosol including extrusion of Na^+ ions out of the cytoplasm, vacuolar compartment -ation of Na^+ ions and restricting the Na^+ inflow. As expected, overexpression of *SOS1* and *NHX1* in transgenic Arabidopsis, cotton, tomato and canola plants shows a significant increase in salt tolerance (Table 1-1, Apse *et al.*, 1999; He *et al.*, 2005; Shi *et al.*, 2003; Zhang *et al.*, 2001; Zhang and Blumwald, 2001). Antisense inhibition of

Table 1-1. Engineered salt stress tolerance through genetic manipulation of plant-source genes.

Gene	Gene Source	Transgenic plant	Method	Traits	Reference
Ion Transporters					
AVP1 (vacuolar H^+-ATPase)	Arabidopsis	Arabidopsis	OX	salt tolerance	Gaxiola *et al.*, 2001
H^+-ATPase (plasma membrane proton pump ATPase)	*N. plumbaginifolia*	tobacco	OX without autoinhibitory domain	increased salt tolerance	Gévaudant *et al.*, 2007
HKT1 (high affinity K^+ transporter)	wheat	wheat	AS	salt tolerance	Laurie *et al.*, 2002
NHX1 (vacuolar Na^+/H^+ antiporter)	Arabidopsis	Arabidopsis, canola, tomato, cotton	OX	salt tolerance	Apse *et al.*,1999; Zhang *et al.*,2001; Zhang and Blumwald, 2001; He *et al.*, 2005
NHX1 (vacuolar Na^+/H^+ antiporter)	*A. gmelini*	rice	OX	salt tolerance	Ohta *et al.*,2002
SOS1 (plasma membrane Na^+/H^+ antiporter)	Arabidopsis	Arabidopsis	OX	salt tolerance	Shi *et al.*,2003
Transcription Factors					
Alfin1	alfalfa	alfalfa	OX	salt tolerance	Winicov, 2000
ABF2(bZIP)	Arabidopsis	Arabidopsis	OX	Enhanced tolerance of drought, salt, heat, oxidative stress, but root elongation inhibited by ABA	Kim *et al.*, 2004
CAP2(AP2)	chickpea	tobacco	OX	tolerance to dehydration and salt stress	Shukla *et al.*, 2006
CpMYB10	*C.plantagineum*	Arabidopsis	OX	drought and Salt tolerance	Villalobos *et al.*, 2004
DREB1A(CBF3)	Arabidopsis	Arabidopsis	OX	freezing, salt, and drought tolerance	Kasuga *et al.*,1999

CBF3	Arabidopsis	rice	OX	increased tolerance to salinity and drought but not to cold	Oh *et al.*, 2005
GmDREB2(AP2/EREBP family)	soybean	Arabidopsis	OX	enhanced tolerance to drought and high-salt stresses	Chen *et al.*, 2007
HARDY(AP2/ERF family)	Arabidopsis	Arabidopsis/ rice	activation tagged mutant/OX	Drought and salt tolerance/Improves drought resistance and water use efficiency	Karaba *et al.*, 2007
OsMYB3R-2	rice	Arabidopsis	OX	increased tolerance to freezing, drought, and salt stress	Dai *et al.*, 2007
OsNAC6	rice	rice	OX	exhibited growth retardation and low reproductive yields. showed improved tolerance to dehydration and high-salt stresses, and increased tolerance to blast disease	Nakashima *et al.*, 2007
SNAC1(NAC TF)	rice	rice	OX	enhances drought resistance and salt tolerance	Hu *et al.*, 2006
StEREBP1(Ethylene responsive element binding protein 1)	potato	potato	OX	enhanced tolerance to cold and salt stress	Lee *et al.*, 2007
Tsi1(EREBP/AP2 TF)	tobacco	tobacco	OX	Salt tolerance	Park *et al.*,2001
Compatible osmolytes					
AtALDH3 (Aldehyde dehydrogenase)	Arabidopsis	Arabidopsis	OX	improved drought, salt and oxidative stress tolerance	Sunkar *et al.*, 2003
BADH(betaine aldehyde dehydrogenase)	barley	rice	OX	improved salt tolerance	Kishitani *et al.*, 2000
IMT1((myo-Inositol-Omethyltransferase)	ice plant	tobacco	OX	drought and salinity stress	Sheveleva *et al.*, 1997
P5CS(Pyrroline-5-carboxylate synthase)	mothbean	tobacco, rice	OX	improved salt and drought tolerance	Kishor *et al.*, 1995; Zhu *et al.*, 1998
ProDH(Proline dehydrogenase)	Arabidopsis	Arabidopsis	AS	improved salt tolerance	Nanjo *et al.*, 1999
ROS related					

Ascorbate peroxidase	Arabidopsis	tobacco	OX	enhanced tolerance to salt, PEG and water stresses	Badawi *et al.*, 2004
FeSOD (superoxide dismutase)	Arabidopsis	tobacco	OX	improved salt and oxidative stress tolerance	Van Camp *et al.*, 1996
glutathione peroxidase (GPX)	*Chlamydomonas*	tobacco	OX	tolerant to salinity (up to 250 mM NaCl) as well as chilling stress	Yoshimura *et al.*, 2004
GST(Glutathione-Stransferase)	tobacco	tobacco	OX	stimulated seedling growth under chilling and salt stress	Roxas *et al.*, 1997
MDAR(Monodehydroascorbate reductase)	Arabidopsis	tobacco	OX	enhanced tolerance against ozone, salt and PEG stress	Eltayeb *et al.*, 2007
Kianses					
AtGSK1 (GSK3/shaggy-like gene)	Arabidopsis	Arabidopsis	OX	improved salt and drought tolerance	Piao *et al.*, 2001
AtNDPK2 (Nucleotide diphosphate kinase)	Arabidopsis	Arabidopsis	OX	enhanced tolerance to salt, cold and methyl viologen treatments	Moon *et al.*, 2003
CIPK15(calcineurin B-like protein-interacting protein kinases)	rice	rice	OX	improved salt tolerance	Xiang *et al.*, 2007
OsMAPK5	rice	rice	OX	increased tolerance to drought, salt, and cold stresses	Xiong and Yang, 2003
MKK2(MAPK kinase)	Arabidopsis	Arabidopsis	OX	increased freezing and salt tolerance	Teige *et al.*, 2004
NPK1(MAPKKK)	tobacco	tobacco	OX	increased tolerance to heat shock, freezing, and salt stress	Kovtun *et al.*, 2000
OsCDPK(Calcium-dependent protein kinase)	rice	rice	OX	improved drought and salt tolerance	Saijo *et al.*, 2000
LEA-like					
DHN-5(dehydrin)	wheat	Arabidopsis	OX	enhanced tolerance to salt and osmotic stress	Brini *et al.*, 2007
HVA1	barley	rice, oat, mulberry	OX	tolerance to drought and salt stress	Xu *et al.*, 1996; Rohila *et al.*, 2002 ; Oraby *et*

					al., 2005 ; Lal *et al.*, 2007
LEA	*B. napus*	Chinese cabbage	OX	enhanced ability to grow under salt and drought stress	Park *et al.*, 2005
Miscellaneous					
ABA2/GIN1(short-chain dehydrogenase/reductase)	Arabidopsis	Arabidopsis	OX	exhibited seed germination delay and more tolerance to salinity	Lin *et al.*, 2007
ABR17(pathogenesis-related protein)	pea	Arabidopsis	OX	germinated better in the presence of salt, cold temperature or both	Srivastava *et al.*, 2006
AtCTL1(chitinase-like protein)	Arabidopsis	Arabidopsis	OX	salt tolerance	Kwon *et al.*, 2007
AtHAL3(flavoprotein)	Arabidopsis	Arabidopsis	OX	improved tolerance to salt and osmotic stresses	Espinosa-Ruiz *et al.*,1999
AtHsp17.6A (Small heat shock protein)	Arabidopsis	Arabidopsis	OX	improved drought and salt tolerance	Sun *et al.*, 2001
AtOAT (Ornithine amino transferase)	Arabidopsis	tobacco	OX	improved NaCl or mannitol tolerance	Roosens *et al.*, 2002
AtRab7 (Vesicle trafficking protein)	Arabidopsis	Arabidopsis	OX	improved salt and osmotic stress tolerance	Mazel *et al.*, 2004
AtVAMP7C(vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptors)	Arabidopsis	Arabidopsis	AS and KO	improved salt tolerance	Leshem *et al.*, 2006
CBL1(calcineurin B-like protein)	Arabidopsis	Arabidopsis	OX	enhanced tolerance to salt and droght but reduced tolerance to freezing	Cheong *et al.*, 2003
CBL4	maize	Arabidopsis	OX	enhanced tolerance to salt at the germination and seedling stages	Wang *et al.*, 2007
Glyoxylase I and Glyoxylase II	*Brassica juncea* and rice	tobacco	OX	improved salt tolerance	Singla-Pareek *et al.*,2003
GlyoxylaseI	*B.juncea*	*B. juncea*	OX	improved salt tolerance	Veena *et al.*, 1999

GmTP55 (antiquitin-like protein)	soybean	Arabidopsis and tobacco	OX	tolerance to salinity during germination and to water deficit during plant growth	Rodrigues *et al.*, 2006
GS2 (Chloroplastic glutamine synthetase)	rice	rice		improved salt tolerance	Hoshida *et al.*, 2000
LTL1(GDSL-motif lipase)	Arabidopsis	Arabidopsis	OX	increases salt tolerance	Naranjo *et al.*, 2006
MBF1a(multiprotein bridging factor 1a)	maize	Arabidopsis	OX	elevated salt tolerance and resistance to fungal disease	Kim *et al.*, 2007
MIPS(l-myo-Inositol-1-phosphate synthase)	halophytic rice	tobacco	OX	capable of growth in 200-300 mm NaCl	Majee *et al.*, 2004
OsISAP1(Zinc-finger protein)	rice	tobacco	OX	tolerance to cold, dehydration, and salt stress	Mukhopadhyay *et al.*, 2004
PDH45 (DNA helicase 45)	pea	tobacco	OX	improved salt tolerance	Sanan-Mishra *et al.*, 2005
PR10(Pathogenesis-related)	pea	canola	OX	enhanced germination and growth in the presence of NaCl	Srivastava *et al.*, 2004
STO (CONSTANS-like protein)	Arabidopsis	Arabidopsis	OX	root growth was increased by 33-70% in the transgenic plants under salt stress	Nagaoka and Takano 2003
ZAT10(C_2H_2-EAR zinc finger protein)	Arabidopsis	Arabidopsis	OX, RNAi and KO	enhanced tolerance to salinity	Mittler *et al.*, 2006

OX, overexpression; KO, knock-out; AS, anti-sense

the wheat high affinity K^+ transporter HKT1 has also resulted in improved salt tolerance (Laurie *et al.*, 2002). It was shown earlier that transgenic plants overexpressing an Arabidopsis vacuolar H^+- pyrophosphatase (AVP1) showed higher tolerance to salinity and drought conditions (Gaxiola *et al.*, 2001).

Transcription factors

Previous researches have identified many transcription factors that are involved in the relaying of stress signals downstream of the sensors to bring about a specific effect. Classically, two pathways have been implicated in the induction of these genes under stress conditions- an ABA independent and an ABA dependent pathway (Fig. 1-2, Xiong *et al.*, 2002b; Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu, 2002). CBFs /DREBs are the transcription factors that bind to CRT/DRE cis elements in the stress induce promoters in an ABA-independent pathway and, ABFs bind to ABRE cis elements in an ABA-dependent pathway (Yamaguchi-Shinozaki and Shinozaki, 2006). Overexpression of *CBF3/DREB1A* confers drought, salt and freezing stress tolerance, however, enhanced expression under the constitutive CaMV35S promoter resulted in severe growth retardation in Arabidopsis (Kasuga *et al.*, 1999). This dwarfing phenotype was not found when the *CBF3* expression is regulated via a stress-inducible *RD29A* promoter (Kasuga *et al.*, 2004). Another group overexpressed Arabidopsis *CBF3* and *ABF3* in rice and observed increased tolerance to salinity and drought without any penalty on rice growth (Oh *et al.*, 2005), which may be partly explained by the differences in the fine regulation of transcript accumulation in the monocot rice and dicot Arabidopsis, although the inner mechanism is unclear. Overexpression of another Arabidopsis ABRE-binding bZIP gene-*ABF2* in Arabidopsis showed tolerance to multiple stresses and altered sensitivity to ABA (Kim *et al.*, 2004). *ABF2* overexpression also promoted glucose mediated inhibition of seedling development (Kim *et al.*, 2004). Dai *et al.* (2007) found that overexpression of a rice R1R2R3 MYB gene, *OsMYB3R-2*, increases tolerance to freezing, drought, and salt stress in transgenic Arabidopsis (Table 1-1).

NAC (NAM, ATAF1,2, CUC2) transcription factors are involved in many diverse plant functions (Olsen *et al.*, 2005). A rice salt-inducible *NAC* (*SNAC1*) gene, when overexpressed in rice, enhanced its tolerance to both drought and salt stress (Hu *et al.*,

2006). Transgenic rice plants overexpressing another rice NAC gene-*OsNAC6*, exhibited growth retardation and low reproductive yields with improved tolerance to dehydration and high-salt stresses, and also increased tolerance to blast disease (Nakashima *et al.*, 2007).

Wang *et al.* (2004) identified a tomato jasmonate (JA) and ethylene responsive factor, JERF3, which is inducible by ethylene, JA, cold, salt and ABA in tomato (Wang *et al.*, 2004). Overexpression of *JERF3* in tobacco results in the induction of pathogen-nesis related genes and enhanced tolerance towards salinity (Wang *et al.*, 2004).

Signaling components

Calcium is known to be an important signal transducer for many stress responsive genes. To date, three major classes of Ca^{2+} sensors have been characterized in plants and they are calmodulin (CaM), CDPKs and CBLs (Yang and Poovaiah, 2003). Several lines of evidence suggest that all these three classes of Ca^{2+} sensors are involved in stress signal transduction (Luan *et al.*, 2002). One CaM was found to bind to a transcription factor MYB2 and was reported to enhance its DNA binding activity. Overexpression of this CaM in Arabidopsis upregulated the transcription of MYB2-regulated genes including *P5CS1* which is known to confer salt tolerance by overproducing proline (Yoo *et al.*, 2005). The mechanism by which calcium regulates sodium homeostasis via SOS pathway has been well elucidated (Zhu, 2002). Xiang *et al.* (2007) systemically characterized 30 rice *CIPK* genes (*OsCIPK01-OsCIPK30*) and studied their responses to various environmental stresses. Three *CIPK* genes, *OsCIPK03, OsCIPK12*, and *OsCIPK15* were overexpressed in rice and transgenic plants showed significantly improved tolerance to cold, drought, and salt stress, respectively. In another study, an *OsCDPK* was overexpressed in rice which showed tolerance to salt and drought stress (Saijo *et al.*, 2000). In addition, MAPK-mediated signaling cascades have also been suggested to play an important role in stress responses by sensing ROS and phosphory-lation of regulatory proteins. Transgenic tobacco plants that constitutively expressed an active *Arabidopsis* MAPKKK, *NPK1*, displayed better tolerance to salt and temperature shocks than wild-type plants (Kovtun *et al.*, 2000). In rice, overexpression of an ABA-inducible *OsMAPK5* increased tolerance to drought, salt, and cold stresses, whereas suppression of *OsMAPK5*

expression by RNAi significantly enhanced resistance to fungal (*Magnaporthe grisea*) and bacterial (*Burkholderia glumae*) pathogens (Xiong and Yang 2003), suggesting a diverse role of some MAPK in abiotic and biotic stress. Similarly, transgenic Arabidopsis that overexpressed a MAPK kinase, *MKK2*, showed tolerance to salt and freezing stresses (Teige *et al.*, 2004).

Osmolytes/compatible solutes

Osmolytes/compatible solutes such as sugars, polyols, amino acids are synthesized and accumulated intracellularly to maintain cell turgor. Over-production of compatible osmolytes such as proline, glycine betaine, trehalose, and mannitol also improve plants' resistance to drought and salt stresses, though the degree to which transformed plants' ability to tolerate salinity stress is not necessarily correlative with the amounts of osmoprotectants attained (Chen and Murata, 2000). In most of these cases, the source of such genes has been *E.coli* and yeast (Chen and Murata, 2000), although a few examples of plant-source genes involved in betaine and proline synthesis have also been reported (Kishor *et al.*, 1995; Kishitani *et al.*, 2000; Nanjo *et al.*, 1999; Zhu *et al.*, 1998).

A problem with the use of compatible solutes to increase salt tolerance is that there are metabolic limitations on the absolute levels of target osmolyte that can be accumulated and polyol over-accumulating transgenic plants show sterility (Apse and Blumwald, 2002). Further studies may be directed towards dissecting the signaling cascades regulating the compatible solute biosynthesis in higher plants.

ROS scavenging

Removal or degradation of ROSs accumulated in cells by a variety of enzymes can also contribute to stress tolerance. For instance, Roxas *et al.* (1997) found that overexpression of glutathione S-transferase (GST) /glutathione peroxidase (GPX) enhances the growth of transgenic tobacco seedlings during chilling and salt stress. Ascorbate peroxidase (APX) is a critical component of the ROS scavenging system in plant cells (Apel and Hirt, 2004). An APX cDNA from Arabidopsis was fused to the chloroplast transit peptide of glutathione reductase (GR) and overexpressed in the chloroplasts. The transgenic plants showed enhanced tolerance to salt and water stress (Badawi *et al.*, 2004). In another report, a *Chlamydomonas* GPX was overexpressed in tobacco in the

cytosol or chloroplasts. The transgenic plants showed increased tolerance to oxidative, chilling and salt stress, indicating a high level of GPX-like protein in tobacco plants functions to remove unsaturated fatty acid hydroperoxides generated under stress and thus, leading to the maintenance of membrane integrity (Yoshimura *et al.*, 2004).

Apart from the various genes discussed above which have been engineered to increase plants tolerance to salt stress as well as to other types of abitoic stresses, there are many other genes which have also been tested in enhancing stress tolerance including salt stress (Table 1-1), although their roles in stress responses are less understood.

However, it should be noted that overexpression of stress responsive genes may not always impart stress tolerance. A stress responsive gene may be involved in stress tolerance or just an effect of stress. Thus, it is not surprising to find that overexpression of a stress responsive gene does not bring any expected trait or even makes the transgenic plants more vulnerable to some types of stress. For instance, it was found that overexpression of a tobacco GST in cotton did not show improved tolerance to salinity, chilling or even to herbicides (Light *et al.*, 2005).

A common problem with supposedly salt-tolerant plants produced by biotechnology is that the assessment of stress tolerance in the lab often has little correlation to that in the field, since saline soil is usually associated with alkaline pH, presence of other sodic salts, low humidity as well as transpiration in the field also influences the assessment. Another problem with those supposedly salt-tolerant plants produced in labs is that salt stress tolerance phenotypes were only observed at specific stages such as seed germination or seedling growth. Therefore, those assessed abiotic stress tolerant plants may not be able to tolerate the true stress consitions in the fields at all. More importantly, salt tolerance needs to be evaluated from an aspect of yield, not just survival and biomass (Flowers, 2004). So far, very few genes have been tested in the fields. Transgenic wheat expressing AtNHX1 was field evaluated. Xue *et al.* (2004) overexpressed *AtNHX1* in wheat and found that the transgenic lines showed improved biomass, increased germination rates and higher grain yields in severe saline conditions.

Although much progress has been made in the past two decades in understanding

salt stress signal transduction mechanisms, there is still a lot unknown or unclear. Therefore, it is necessary to understand how plants respond to stress in a systematic way before we apply the results gained in the lab to crop plants to engineer stress tolerance. Arabidopsis will continue to play a critical role in the foreseeable future, not only in the understanding of biological mechanisms of abiotic stress tolerance, but also in providing a facile means for testing in biotechnology.

Conclusions and future perspectives

Salt stress presents an increasing threat to agriculture in many parts of the world. A major challenge faced by plant scientists is to increase crop productivity while maintaining the sustainability of agricultural practices. It is clear that improving crop tolerance to abiotic stresses will play an important role for fully exploiting the productivity potential of crops. In the past few decades, worldwide researchers have produced an enormous amount of information regarding to the different components of salt signaling pathway, the cross talk between salt and other abiotic stresses as well as the utilization of various genes to engineer salt-tolerant plants. So far, many identified key molecular factors that can be used for genetic engineering of salt-tolerant plants include overexpression of specific transcription factors, characterization of dehydrin proteins, overproduction of osmoprotectants, expression of water channel proteins and ion transporters. In recent years, small RNAs such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are small noncoding RNAs working as important regulators of mRNA degradation, translational repression, and chromatin modification, have been shown to play important roles in plant growth and development and stress tolerance (Sunkar and Zhu, 2004; Borsani *et al.*, 2005; Fujii *et al.*, 2005; Phillips *et al.*, 2007 ; Sunkar *et al.*, 2007). Overexpression of these miRNAs and characterization of their phenotypes would shed light on their biological functions. Recently, researches on the new model plant, salt cress (*Thellungiella halophila*) have provided new insights into salt and cold tolerance mechanisms in this small winter annual crucifer (Inan *et al.*, 2004; Volkov *et al.*, 2004; Vera-Estrella *et al.*, 2005; Kant *et al.*, 2006; Volkov and Amtmann, 2006; Wang *et al.*, 2006). Salt cress has a short life cycle and a small genome (~230 Mb) with high sequence identity (average 92%) with Arabidopsis. Salt

cress is an extremophile native to harsh environments and can reproduce after exposure to extreme salinity (500 mm NaCl) or cold to -15 °C (Inan *et al.*, 2004). Future priorities can be directed towards the identification of molecules connecting pathways and of key components in each pathway. Past researches in this field suggest that our understanding of plant stress tolerance can be greatly refined by thorough characterization of individual genes and assessing their contribution to stress tolerance. Such experiments indicated that many individual genes appear to have some positive impact on stress tolerance; mainly master switches such as transcription factors or their upstream signaling molecules are promising candidate genes for biotechnology-ical approaches. Therefore, a deeper understanding of the transcription factors and their regulated target genes, the products of the major stress responsive genes and cross talk between divergent signaling components should remain an area of intense research activity in the near future. The knowledge generated through these studies can be applied to engineer transgenic plants that can tolerate stress conditions without showing any growth and yield penalty.

Objectives

The short term objectives of my research are to identify a complete set of salt responsive genes through full-genome microarray profiling and 2D-gel electrophoresis of NaCl-treated *Arabidopsis* roots; and to characterize a few novel salt responsive transcription factor genes through characterizing their expression and function using a variety of techniques including reverse genetics. The long-term goal is to develop salt-tolerant plants which may also be tolerant of other abiotic stresses like drought.

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Chapter 2: Microarray analysis of Arabidopsis root transcriptome changes in response to salt stress

Introduction

Roots are the primary site of perception and injury for several types of water-limiting stress, including salinity and drought. In many circumstances, the stress-sensitivity of the root limits the productivity of the entire plant (Atkin *et al.*, 1973; Steppuhn and Raney, 2005). The physiological significance of roots is belied by their relative structural simplicity as compared to other organs: roots are largely lacking in some major metabolic pathways such as photosynthesis, and have a stereotypical morphology that is conserved across taxa and throughout the life cycle of individuals. This combination of physiological relevance and structural simplicity has made roots good targets for functional genomic studies. For example, detailed transcriptional profiles have now been resolved to single cell types within roots, and these are now being integrated into regulatory circuits and networks (Birnbaum and Benfey, 2004).

Salinity treatment of plants is also an attractive experimental system. NaCl treatments are easy to apply in laboratory settings, with dosage and timing controlled more precisely than with other major abiotic stresses such as chilling, freezing, and dehydration. So far, at least nine published reports used microarrays to analyze the response of Arabidopsis to NaCl. However, most of these studies have analyzed either cell cultures or whole plants, rather than specific tissues (Seki *et al.*, 2002a; Taji *et al.*, 2004; Takahashi *et al.*, 2004; Gong *et al.*, 2005; Kamei *et al.*, 2005; Ma *et al.*, 2006). Of the previous studies that analyzed roots specifically, none used microarray probe sets representing more than 8,100 of the originally predicted 25,498 genes in the Arabidopsis genome (Arabidopsis Genome, 2000; Chen *et al.*, 2002; Kreps *et al.*, 2002; Maathuis *et al.*, 2003). Although Affymetrix microarrays containing probes for at least 22,591 Arabidopsis genes have been used to profile NaCl responses specifically in

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roots, these data were generously deposited to public databases, but without detailed description or analysis in the primary literature. Thus, the absence of available, comprehensive transcriptomic data describing the response of Arabidopsis roots to NaCl treatment, in combination with the potential applications of these data in molecular physiology and systems biology, motivated us to conduct this research.

Materials and Methods

Plant growth and stress treatment

We sowed surface-sterilized wild-type seeds of *Arabidopsis thaliana* (ecotype Col-0) on (3.5 mm diameter) agar plugs arrayed on custom-made polypropylene rafts covered by 0.5 mm nylon mesh (Northwest Graphic Products Ltd, Edmonton) from beneath, and cold stratified them at 4°C for 2 d. Each plug consisted of ½ X Murashige and Skoog (MS, Sigma) basal salts, 1% sucrose, and 0.7% Phytagar (Invitrogen), and each raft held approximately 30 seeds, spaced ca. 15 mm apart. Rafts were transferred into opaque plastic tanks containing 1 L of autoclaved ¼ X MS solution (pH5.7) in a growth chamber at 22°C, 70% relative humidity, and light (~125 $\mu E\ m^{-2}\ s^{-1}$) on a 16h light/8h dark cycle. The solution in the tanks was aerated and replaced with fresh, autoclaved ¼ X MS solution every 4 days to limit growth of microorganisms. At 20 days after stratification (DAS), seedlings were treated with ½ X MS medium (pH5.7) supplemented with 150 mM NaCl beginning at 8:30AM (1.5 h after the start of the light cycle), and roots were harvested at 6, 24 and 48 h post-treatment, and were frozen in liquid nitrogen and stored at -80°C. Untreated controls were harvested in parallel, at the same time points. Approximately 150 plants were harvested and pooled for each combination of time point and treatment, in each biological replicate. This process was repeated in three biologically and temporally independent replicates.

Physiological analyses

Proline concentrations were measured using the method of Bates (Bates *et al.*, 1973), with L-proline (Sigma) as a standard. Anthocyanin concentrations were determined according to Macinelli and Schwartz (1984).

Microarray preparation, hybridization and data extraction

QiagenOperon *Arabidopsis* 70-mer oligonucleotide probes (Array-Ready Oligo Set

Version 1.0) representing 26, 029 genes, plus additional probes for quality control were printed on superamine aminosilane-coated slides (Telechem) in a format of 12 X 4 subarrays with each containing 24 X 24 non-replicate spots. Probe spotting followed previously described methods (Wang *et al.*, 2003)

Total RNA was isolated from both control and stressed root tissues using the RNeasy Plant Midi kit (Qiagen), and was concentrated by Microcon YM-30 (Millipore) to ~ 1.2 μg/μL and quantified by NanoDrop ND-1000 Spectrophotometer. RNA integrity was checked on a formamide denaturing agarose gel. 5 μg total RNA was used to synthesize cDNAs using SuperscriptII (Invitrogen) with RT polyA-capture primers in 3D Array900 kit (Genisphere). Before hybridizations, microarray slides were rehydrated over a 60°C water bath for 5 seconds, snap-dried on a 65°C heating block for 5 seconds and allowed to cool for 1 min. This process was repeated for a total of five times. Then the slides were UV cross-linked by exposing the slides label-side up to 120 mJ for 5 min. Slides were then washed in 1% SDS for 5 minutes, dipped into 95% ethanol for 30 seconds before dried by centrifugation at 1,000 rpm for 1 min. A two-step hybridization was performed, i.e., cDNA from both control and treated tissue were hybridized together at 55°C overnight under a 24X 60 mm cover slip (Fisher Scientific) in a sealed humid 50 ml centrifuge tube. Secondary hybridization with Cy3 and Cy5 dendrimers were performed at 55°C for 4 h and washes were done according to the manufacturer's instruction. Slides were scanned immediately using ArrayWorxe (Applied Precision) and transformed into .tiff images. To avoid bias in the microarray evaluation as a consequence of dye-related differences in labelling efficiency and/or differences in recording fluorescence signals, dye labelling for each paired sample (stressed/control) was reversed in two individual hybridizations. Therefore, two hybridizations were performed for each of three independent biological replicates at each time point.

Microarray data analysis

Background-subtracted spot intensities were measured by Spotfinder v3.0, and normalized by the Lowess method in MIDAS v2.19 (Saeed *et al.*, 2003). Spots were defined as detectable above background if their signal intensity in at least one channel was greater than two standard-deviations above the mean signal intensities of all blank and

randomized negative control spots, and if they were detectable in at least two-thirds of arrays at any one time point. Next, MeV(v3.1) was used to find the differentially expressed genes between control and NaCl treatment. To do so, the integrated spot intensities were loaded into MeV and we used a two-channel spot intensity cut-off value of 1,000. Significance analysis of microarrays (SAM) contained in the was applied to find genes for which the fold change differed significantly from zero in a multiclass test (with inferred values at time point 0), using a false discovery rate (FDR) of ≤ 5% (Tusher *et al.*, 2001). Fold changes (treated/control) were calculated in log2 scale. Assignment of genes to temporal expression profiles and detection of statistically enriched gene families within each profile was conducted using STEM (Short Time Series Expression Miner) software, with maximum number of model profiles set to 16, and maximum unit change set to 3 (Ernst and Bar-Joseph, 2006). The input data for STEM analysis consisted of all normalized, intensity-filtered data points identified by SAM analysis as statistically distinct from 0. The p-values derived from STEM analysis were corrected for multiple hypothesis testing using a FDR of < 5% (Draghichi, 2003).

Quantitative PCR

Aliquots of the same RNA samples used for microarray analysis were also used for real-time PCR. Reverse transcription (RT) was performed with 2 μg of total RNA pretreated with DNase I (Ambion) to obtain cDNA with SuperScript II and Oligo$(dT)_{12-18}$ (Invitrogen) as the primer in a 20 μL reaction volume. Each cDNA sample was diluted 1:4 in sterile ddH_2O, and 1 μL of this dilution was used as template for qPCR. Primers for the PCR reactions were designed by PrimerSelect 5.00 of DNAStar (DNASTAR Inc) to have a Tm of ~ 60°C and an optimal annealing temperature of 53-55 °C with the length of the amplicons between 80 and 200 bp. Real-time PCR was performed with QuantiTech PCR SYBR Green I Kit (Qiagen) in 20 μL reactions using the LightCycler 1.5 System (Roche Diagnostics) according to the manufacturer's instruction. Each PCR reaction contained 1 μL of cDNA, 0.5 μM of each of the primers and 10 μL of master mix. The initial denaturing time was 15 min at 95°C, followed by 35 cycles consisting of 94°C for 15 s, 53 °C for 30 s, and 72°C for 20 s with a single fluorescence measurement. A melting curve (65°C–95°C with a

heating rate of 0.05°C s^{-1} and a continuous fluorescence measurement) was run after the PCR cycles followed by a cooling step at 40°C.

For relative quantification, amplification efficiencies (E) for each gene were determined as follows: a portion of cDNAs transcribed from 5 µg of total root RNA was diluted with sterile ddH_2O to be 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Standard curves for each gene was performed using 1 µL of the original and diluted cDNAs to cover the range of all template concentrations. Real-time PCR efficiencies (E) were calculated from the given slopes in the LightCycler software of the standard curves according to the equation: $E = 10^{-1/slope}$. Crossing points (CP), defined as the point at which the fluorescence rises above the background fluorescence was determined using the "Fit Point Method" in the LightCycler software 3.5.3 (Roche Diagnostics). Gene-specific PCR efficiency was used to calculate the expression of target genes relative to the expression of UBQ10 reference gene according to the format: Ratio = $(E_{target})^{\Delta CP_{target(control - sample)}} / (E_{ref})^{\Delta CP_{ref(control - sample)}}$ (Paffl 2001).

The genes tested for expression levels by quantitative PCR were as follows: At1g44830 (AP2/EREBP), forward primer (FP), 5'-AGTTGGGGTTCATGGGT TTCA-3', reverse primer (RP), 5'-ACATAGGAGTGCTGCGTCGTAGG-3'; *WRKY17* (At2g24570), FP, 5'-CAGCGAGGAAACACGTGGAAAGAGC -3', RP, 5'-CAAGC-CGAACCAAACACCAAACCAC -3'; *MYB15* (At3g23250), FP, 5'-AAATACTTGG-CAATAGATGGTCAG -3', RP, 5'-ACGGATTCAGATTTTGGTTTAGTA-3'; *XTH18* (At4g30280), FP, 5'- AGGCTCCTTTCACCGCTTTCTA -3', RP, 5'- ACTC TGTACCCCTTTCATTCTTGTCTG -3'; At4g01550 (*NAC*), FP, 5'- AGAGAAGAA GAGCTGGTTTATTACA -3', RP, 5'- TTCATCGCCGGGTTCAAGT -3'; At2g04070 (MATE), FP, 5'- TCTTTCTGGTTTTTCGCTATGACACT -3', RP, 5'- ATTGCCGC TGATGG-GACTC -3'; At4g26410, FP, 5'-GAGCTGAAGTGGCTTCCATGAC-3', RP, 5'-GGT-CCGACATACCCATGATCC-3'; *CYP83B1/SUR2* (At4g31500), FP, 5'-ACTCTTGAC-CC TAACCGCCCTAAAC-3', RP, 5'-TGCAGCCGCCGTGTCAGT-3'; *bHLH92* (At5g43650), FP, 5'-CACGGAACTGAGGATGATGTC-3', RP, 5'-GTC GCCGGCTT-CTTTCCTTCTCT-3'. *WRKY25* (At2g30250), FP, 5'- TGGTTCTTCC GGCGTTGACTGTTA-3', RP, 5'-GTGAAATCGGAAGAG-GTGGTGGTTG-3'; *WRKY33* (At2g38470), Fwd, 5'-TTACGCCACAAACAGAGCAC-3', Rvs, 5'-CCAA

AAGGCCCGGTATTAGT-3'; At3g17860 (*ZIM*), FP, 5'- GGCTCCAACAGTGGCAT TACCTCT -3', RP, 5'-TATGGGGATACGCTCGTGACCCTT -3'; *PER21* (At2g37130), FP, 5'- TGCGAGAGACGGTATTGTCA-3', RP, 5'- TCTCCCAAGTA GCTCCCTCT -3'; *PER27* (At3g01190), FP, 5'- CGCAATGGTTGCACTTGA-3', RP, 5'-TGAGCGAAA-ATCGCTGATAA -3'; *PER69* (At5g64100), FP, 5'- CTCTTG TTGGCGGACACA-3', RP, 5'- GTCGATTGATGGGTCAGGTT -3'.

The internal control gene was *UBQ10* (At4g05320), which is a suitable reference gene for abiotic stress analysis (Czechowski *et al.*, 2005). The primer sequences are 5'-TAC TCT TCA CTT GGT CCT GCG TCT TC-3' (FP) and 5'- GGC CCC AAA ACA CAA ACC ACC AA-3' (RP). qPCR for each gene was done on three biological replicates with duplicates for each biological replicate. The relative transcript level was determined for each sample, normalized using the *UBQ10* cDNA level, and averaged over the six replicates.

Results and Discussion

Whole-plant responses to salt treatment.

We applied a salt-shock treatment to 20 DAS (days after stratification) Arabidopsis plants by supplementing their hydroponic growth medium with 150 mM NaCl (Fig 2-1). This concentration of NaCl has been used in several previous gene expression studies, since it induces a moderate stress response and is not acutely lethal (He *et al.*, 2005; Ma *et al.*, 2006). Indeed, after application of 150 mM NaCl, we observed visible signs of stress including loss of turgor. However, even after 48h of exposure to media supplemented with 150 mM NaCl, nearly all of the treated plants recovered and resumed growth when transferred into NaCl-free hydroponic medium (data not shown). Obviously, these experimental conditions differ from those experienced by soil-grown plants, especially field-grown crops, where multiple stresses and nutrient limitations can occur simultane-ously (Mittler, 2006). Nevertheless, we expect that many stress response pathways will be conserved across treatments. This assumption, along with the precise experimental control afforded by laboratory hydroponics, justifies the use of hydroponics as model system for salinity stress.

To further define the physiological state of the plants that we subjected to

transcriptional profiling, we monitored the accumulation of two stress-inducible metabolites, namely anthocyanin and proline (Yoshiba *et al.*, 1997). Recent evidence suggests that anthocyanins may mitigate photooxidative injury by efficiently scavenging free radicals and reactive oxygen species (Gould, 2004). Anthocyanin concentrations increased rapidly in leaves of stressed plants, with the absorbance at diagnostic wavelengths increasing nearly three-fold in the first 6h after treatment (Fig 2-1). These pigments continued to accumulate, with a five-fold increase observed at 24 h after treatment. Interestingly, the concentration of anthocyanin began to decrease after 24 h of stress, perhaps due to catabolism of the accumulated pigment and acclimation to the stress conditions, resulting in levels of anthocyanin at 48h post-stress

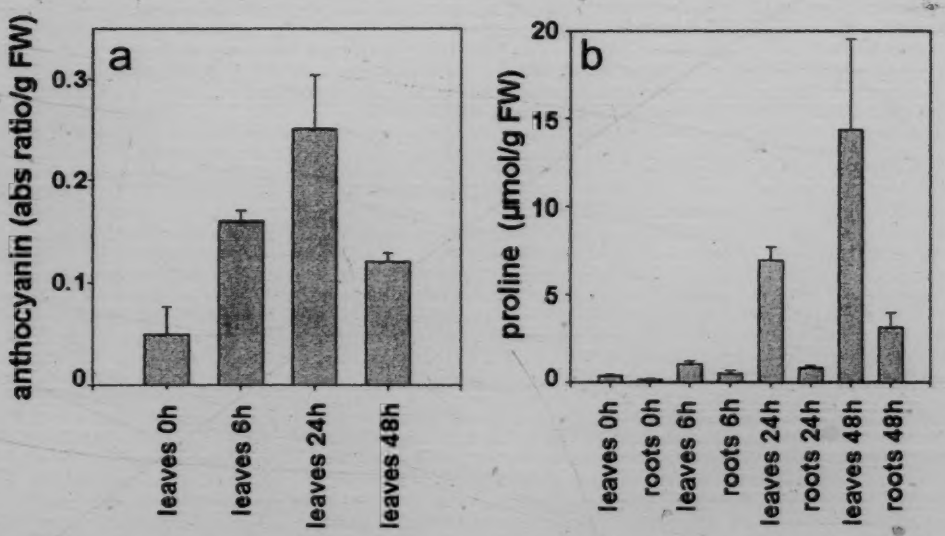

Fig 2-1. Accumulation of anthocyanin and proline in Arabidopsis plants in responses to NaCl treatment. a) Anthocyanin accumulation in Arabidopsis leaves. Anthocyanin concentration is reported as an absorbance ratio: (A530-1/4*A657)/g fresh weight. b) Proline accumulation in roots and leaves of Arabidopsis plants. Arabidopsis plants were mock treated or exposed to 150mM NaCl for 6, 24 and 48 h. The means of six experiments ± S.D. are shown.

that were slightly lower than levels observed at 6h. Proline concentration increased rapidly in both the leaves and roots of NaCl-stressed plants until at least 48h following NaCl treatment (Fig 2-1). We observed a 3-fold increase in proline concentration in roots after 6h, and an 18-fold increase after 48h. A similar pattern of increase, with

higher absolute concentra-tions of proline, was also observed in shoots. The accumulation of these metabolites indicates that these plants were actively mounting a stress response at the time of transcriptional profiling.

General transcriptomic responses

To characterize the effect of NaCl treatment on gene expression in Arabidopsis roots, we extracted RNA from control and stressed root samples at 6, 24, and 48h following treatment, and analyzed these in paired, dye-reversed hybridizations on spotted 70-mer oligonucleotide microarrays consisting of probes representing 23,687 unique genes identified by Arabidopsis Genome Initiative (AGI) locus identifiers. We identified probes for 7,217 unique genes whose treated:untreated $\log_2$ expression ratio differed significantly from 0 at one or more time points, according to the Significance Analysis of Microarrays (SAM) algorithm, with a false discovery rate (FDR) of less than 5% (Tusher *et al.*, 2001). Using a 2-fold cutoff, the number of salt up-regulated genes is 1540, 1444 and 1642 for 6, 24 and 48 h time points, respectively, whereas the number

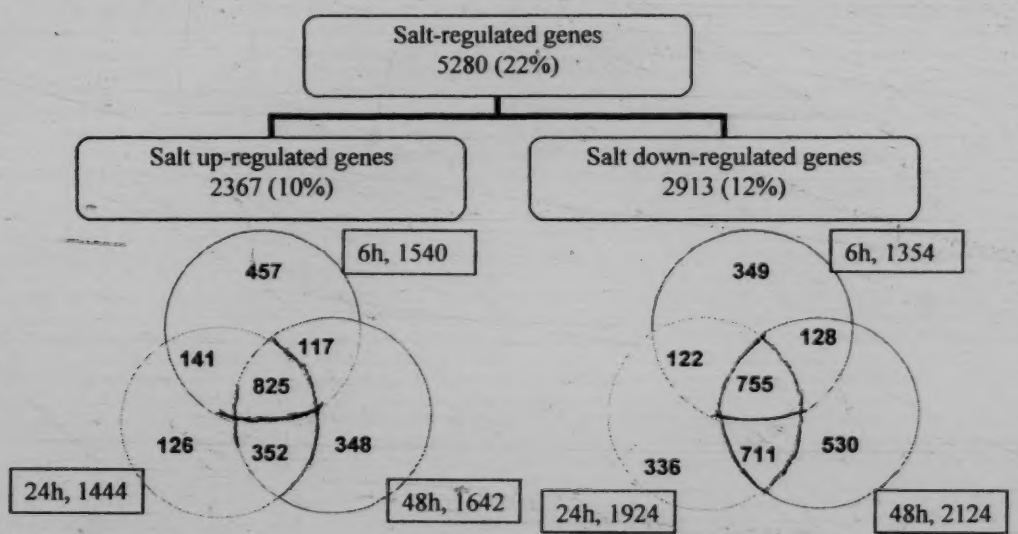

Fig. 2-2. Venn diagrams of salt-regulated genes. Percentages in parentheses were calculated with the total numbers of genes assigned with AGIs (23, 687). Figures in rectangles indicate salt treatment hours (h) and total number of salt-regulated genes at each time point. Only genes showing ≥ 2-fold between treatment and control with a FDR≤ 5% in SAM were included here. Genes/probes that passed filterings (signal intensity above background on more 4 slides and FDR ≤5%) but have no AGI assigned are not listed here.

of salt down-regulated genes is 1354, 1924 and 2124 for 6, 24 and 48 h time points, respectively. In total, 5280 unique genes with AGI assigned were identified to be salt-regulated (Fig. 2-2). Among these statistically significant expression ratios, probes representing 2,367 unique genes showed a NaCl-induced increase in transcript abundance of at least 2.0 fold at one or more time points. Conversely, probes representing 2,913 unique genes showed a decrease in abundance of at least 2.0 fold or more. Thus, transcript accumulation for at least 10% of Arabidopsis genes was strongly (i.e. >2.0 fold) induced, and transcripts for at least 12% of genes were repressed at one or more time points, in response to NaCl-treatment. The proportion of NaCl-responsive genes we observed is somewhat higher than previously reported estimates based on cDNA microarrays, likely reflecting the higher specificity of the oligonucleotide-based arrays (Seki *et al.*, 2002a).

Validation of microarray results

We compared our results to publicly available microarray data from the AtGenExpress project, which used Affymetrix ATH1 arrays to describe the transcriptome of hydroponically grown Arabidopsis roots exposed to a 150 mM NaCl-shock for up to 24h (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp). Qualitative trends in expression ratios are highly conserved between these datasets. Quantitatively, we observed a Pearson correlation co-efficient of 0.77 (6h) and 0.69 (24h) when comparing the ATH1 expression values with our signal-intensity filtered ratios. The transcriptomic responses we describe here are therefore generally reproducible across platforms and between laboratories. Moreover, probes for at least 3,277 genes were deposited on our spotted microarray that are not present on the ATH1 array, of which 730 were significantly responsive to NaCl treatment. For example, the gene with the highest-fold induction (mean ratio=7.4 ($\log_2$)) in our analysis is transcription factor *bHLH092* (At5g43650), but this gene is not represented on the ATH1 array. Thus, although the Affymetrix ATH1 array and the Operon 1.0 70-mer probe set are each commonly described as "whole-genome", it is clear that either platform alone is deficient in the detection of hundreds of potentially significant genes, although in combination, they provide a nearly complete representation of the predicted transcriptome.

To provide further validation of our microarray data, we performed quantitative RT-PCR analysis on specific transcripts (Table 2-1). We selected 15 genes representing different functional categories, which according to our microarray analysis increased, decreased, or remained essentially unchanged in terms of transcript abundance following salt-shock. For all of these genes, the expression ratios measured by qRT-PCR and by microarray were highly correlated (r= 0.91, 0.92 and 0.88 for 6, 24 and 48h treatment, respectively). These results help to confirm the general accuracy of the microarray data we present here. The functional significance of the NaCl-responsive genes that were validated by qRT-PCR is discussed in more detail below.

Table 2-1 Comparison of qRT-PCR and microarray results for selected genes.

AGI#	annotation	microarray (log2 ratio)			qRT-PCR (log_2 ratio)		
		6h	24h	48h	6h	24h	48h
At1g44830	AP2/ERF	4.37	5.42	4.89	6.05	6.37	6.47
At5g43650	bHLH92	7.21	7.58	7.45	11.05	11.93	11.87
At4g31500	CYP83B1/SUR2	-2	-1.89	-1.43	-1.84	-1.6	-1.22
At4g26410	expressed protein	0.01	0	0.04	-0.3	-0.23	-0.27
At2g04070	MATE	3.77	5.47	5.35	3.1	4.92	5.27
At3g23250	MYB15	4.05	3.25	1.31	5.39	5.67	5
At4g01550	NAC	1.93	3.35	3.21	2.46	4.87	5.65
At2g37130	PER21	-1.73	-1.56	-0.98	-1.32	-1.4	-0.69
At3g01190	PER27	-0.37	-0.02	0.14	-2.96	-1.21	-0.18
At5g64100	PER69	-1.78	-2.69	-1.8	-1.85	-2.4	-2.2
At2g24570	WRKY17	3.74	1.94	1.16	3.84	2.15	1.64
At2g30250	WRKY25	2.59	4.04	4.49	3.49	6	6.23
At2g38470	WRKY33	4.02	4.42	3.58	4.81	5.58	4.64
At4g30280	XTH18	2.54	1.3	1.57	2.65	1.58	2.5
At3g17860	ZIM	2.28	0.65	-0.84	2.42	1.02	-0.97

Genes representing a variety of expression patterns and functional categories were selected from the microarray dataset for further validation using qRT-PCR. Values shown are the mean of at least four independent measurements (microarray data), or three independent measurements (qRT-PCR).

Classification of NaCl-responsive transcripts

We used the STEM to summarize our filtered microarray data, by clustering it into 16 distinct temporal expression patterns (Fig 2-3). The algorithms implemented in STEM are designed specifically for microarray experiments that sample only a few time points, such as ours. STEM accordingly minimizes the potential for over-fitting that can occur when some other clustering methods are applied to time course data, and also

facilitates the identification of over-represented functional categories within each cluster, as described below.

The predominant temporal expression patterns detected by STEM analysis show that changes in transcript abundance can be detected within the first six hours following treatment for the majority of the 5,463 NaCl-responsive genes represented in the profiles (Fig 2-3.). At subsequent time points (i.e. 24h, 48h) the response generally either continued along the same trajectory (Fig 2-3 a,i), or remained close to the level of induction or repression observed at 6h (Fig 2-3 b,j). Another subgroup of genes had a peak response at 6h, which returned towards untreated levels of transcript abundance either gradually (Fig 2-3 c,k), or more rapidly (Fig 2-3 d,e,l,m). It is notable that a significant number of these genes show opposite patterns of induction and repression at 6h as compared to 48h (Fig 2-3 d,l). Still other genes that were responsive at 6h showed a peak change in expression at 24h (Fig 2-3 f, n), or, surprisingly, a moderate dampening of the response at 24h, and a renewed intensity of response at 48h (Fig 2-3 g,o). Finally, in contrast to all of the patterns discussed so far, which began with a marked response at 6h, a limited but significant number of genes showed relatively little response until the 48h time point (Fig 2-3 h,p). Thus, although the majority of genes are responsive by 6h of treatment, dynamic patterns of gene expression are also detected after 24h and 48h of treatment. These observations indicate that different regulatory networks and effectors are active at each of these different periods following the perception of stress.

STEM was used to identify categories of functionally and/or structurally related genes that are enriched (i.e. statistically over-represented at FDR<5%) in one or more of the clusters described above. Preliminary analysis using Gene Ontology consortium categories (data not shown) was used to guide the selection of a detailed list of gene families and superfamilies from primary literature sources, as well as from curated databases including KEGG (Kyoto Encylcopedia of Genes and Genomes), AraCyc, and TAIR (The Arabidopsis Information Resource) (Kanehisa and Goto, 2000; Rhee *et al.*, 2003; Zhang *et al.*, 2005). Most broadly, the functional themes represented by NaCl-responsive transcripts can be divided into effectors and regulators. According to this analysis, the most prominent effectors of the NaCl stress response are genes involved in

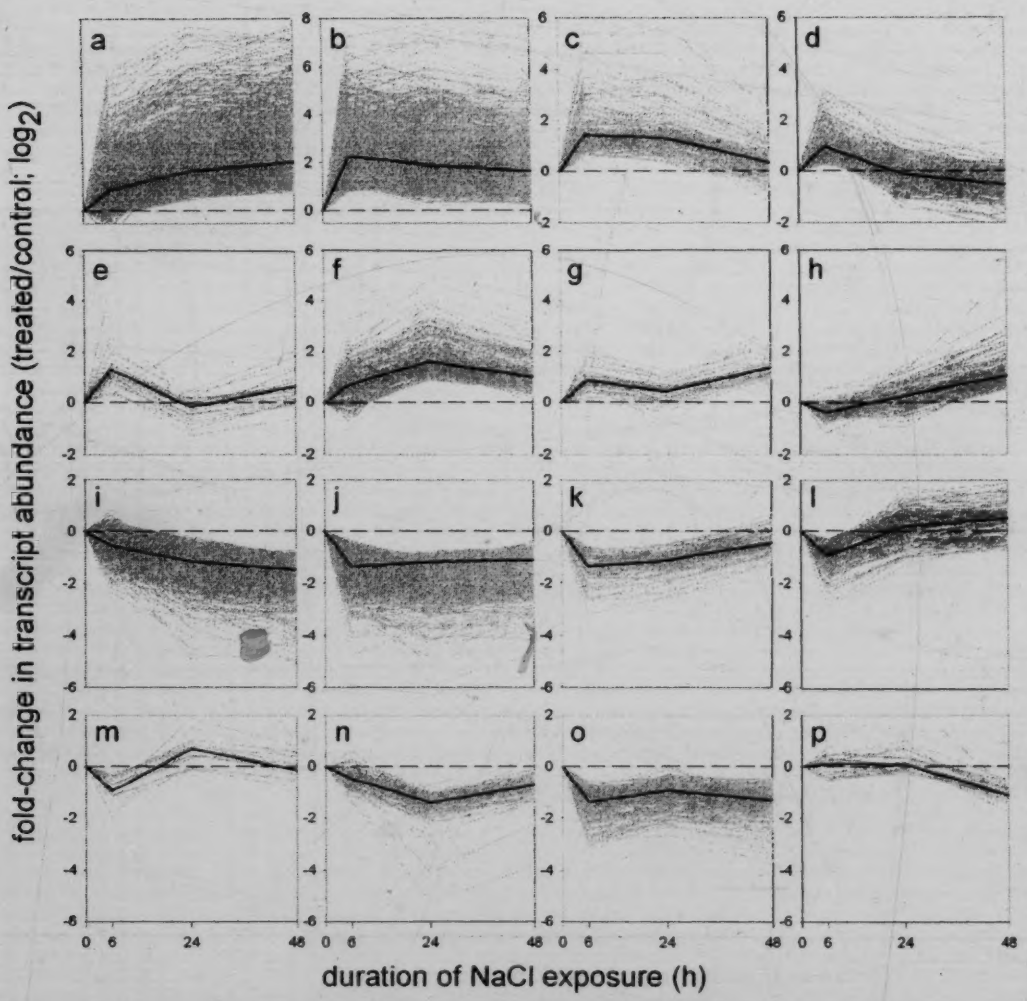

Fig. 2-3. NaCl-responsive transcripts grouped according to temporal expression profiles. Microarray expression data that had been previously filtered for significance based on signal intensity and SAM statistical analysis, was divided into 16 distinct temporal profiles, using STEM software. Each of the profiles (labelled a-o) is represented as a different plot, with mean expression ratios (log_2; treated:control) for each of the assigned transcripts at each time point plotted in grey. The median of all assigned expression ratios in each profile is plotted in black. To emphasize that the y-axis range is different between plots, a dashed line corresponding to a log_2 expression ratio of 0 is also shown in each plot.

detoxification, transport, protein turnover, energy metabolism, and production of osmoprotectants. The major classes of regulators detected are signal transduction

components, transcription factors, and hormone related genes. Each of these broad themes has been previously reported in association with abiotic stress responses (Sahi *et al.*, 2006). The analysis we present here highlights specific NaCl-responsive genes and groups of genes within these categories. Tables 2-2 to 2-6 show groups of functionally and/or phylogenetically related genes that, according to STEM analysis, were significantly over represented (FDR<5%) in one or more STEM profile (Fig 2-3), as well as a limited number of other groups of genes mentioned in the text, for comparison. The tables also include the number of genes within each category that were up- or down-regulated more than 2-fold, although this 2-fold threshold is not a criterion *per se* in STEM analysis.

Osmoprotectants

Osmoprotectants including proline and trehalose, and small hydrophilic proteins such as dehydrins help to maintain hydration of cellular components during osmotic stress (Alsheikh *et al.*, 2005). Genes regulating levels of osmoprotectants are highly stress responsive and were among the first stress-inducible transcripts reported in the literature (Gilmour *et al.*, 1992; Yamaguchi-shinozaki *et al.*, 1992; Verbruggen *et al.*, 1993; Welin *et al.*, 1994). As expected, we observed induction of many of these genes in our NaCl-treated tissues (Table 2-2). Two proline biosynthetic genes were induced (*P5CR*, At5g14800; *P5CS*, At2g39800), consistent with the observed increase in proline abundance (Fig 2-1). We also observed transcript-level induction of a large proportion of genes in the trehalose biosynthetic pathway including 7 of the 8 trehalose phosphatases detectable on our array, as well as some (but not all) dehydrins (Table 2-2). Although trehalose has been shown to act as an osmoprotectant in bacteria and fungi, recent studies have suggested that this disaccharide acts as a signalling molecule in plants, noting that its intracellular concentration is too low to be an effective osmoprotectant (Avonce *et al.*, 2004).

Reactive oxygen network

NaCl treatment, like many environmental stresses, generates reactive oxygen species (ROS) (Mittler *et al.*, 2004). These ROS and the products of their reactions with other cellular components (e.g. peroxidized lipids) are generally toxic. It is therefore not surprising that plants have evolved large gene families for detoxification of ROS and

Table 2-2. NaCl-responsive transcripts related to osmoprotection, detoxification, and transport.

family, group, or pathway	number of genes in genome	on array	signal above bkgnd.	>2 fold up	>2 fold down	assignment to STEM profiles (Fig. 2-3) cluster:# of genes
osmoprotectants						
proline	5	5	5	2	1	a:1;b:1;j:1
trehalose biosynthesis*	22	21	15	8	1	a:4;b:4;c:1
trehalose phosphatase*	11	10	8	7	0	a:3;b:4
dehydrin*	10	10	6	4	1	b:1;c:2;h:1;i:1
ROS-response network						
alternative oxidase*	6	6	4	2	0	b:2
ascorbate peroxidase	9	8	6	1	1	b:1;h:1;o:1
blue copper protein	9	8	5	1	1	b:1;i:1;n:1
dehydroascorbate reductase	5	2	1	0	1	i:1
ferritin*	4	4	3	2	0	a:2
glutaredoxin*	27	26	8	3	3	a:3;i:1;j:2
glutathione peroxidase	8	8	4	0	1	j:2
glutathione reductase*	5	4	4	0	0	m:1
monodehydroascorbate reductase	5	5	4	2	0	a:1;c:1
NADPH oxidase*	10	10	4	1	2	c:1;i:1;k:1
NADPH oxidase-like	9	8	4	0	3	i:2;j:1
peroxiredoxin	11	10	5	0	2	j:2
superoxide dismutase	8	8	3	0	3	i:1;j:2
thioredoxins	32	31	20	5	7	a:1;b:2;c:1;f:1;i:2;j:3; l:1;o:1
class III peroxidase*	73	71	45	10	27	a:6;b:3;c:1;d:1;i:3;j:17 k:4;l:1;o:3
glutathione-S-transferase*	53	49	37	19	7	a:4;b:9;c:1;d:3;e:1;f:1; h:2;i:3;j:2;l:2;n:1;o:1
GST-tau family*	28	26	20	13	3	a:1;b:9;c:1;d:3;i:1;j:1; n:1
transporters						
aquaporin*	35	33	24	1	18	a:1;i:4;j:12;n:1
V-Type ATPase*	22	18	14	0	4	j:4
antiporter	70	66	25	5	9	a:4;c:1;i:2;j:4;k:1;l:2
Na+/H+ exchanger*	8	8	6	4	0	a:4
sugar transporter*	48	47	23	11	4	a:6;b:3;d:1;f:1;i:1;j:2; o:1
ABC transporter*	94	88	29	10	5	a:6;b:2;d:3;f:1;g:1;i:1; k:1;l:2
LeOPT1*	51	48	22	10	8	a:4;b:6;i:3;j:3;o:2
MATE*	56	51	26	13	1	a:4;b:6;c:1;d:1;f:1; i:1

Groups of genes that are significantly enriched in at least one STEM profile at a FDR of <5% are marked with an asterisk (*). The final column details the frequency of assignment of individual genes to specific STEM profiles identified by corresponding letters in Fig 2-3.

Functional categories in this table are defined according to the following sources: proline and trehalose biosynthesis (Zhang *et al.*, 2005); dehydrins (Alsheikh *et al.*, 2005); ROS-network (Mittler *et al.*, 2004); class III peroxidases (Tognolli *et al.*, 2002; Rhee *et al.*, 2003), GSTs (Rhee *et al.*, 2003); MATE transporters (Rogers and Guerinot, 2002); aquaporins(Rhee *et al.*, 2003; Boursiac *et al.*, 2005); all other transporters (Ward, 2001; Rhee *et al.*, 2003).

their by-products. At least 148 enzymes have been defined as part of the ROS scaven -ging network of Arabidopsis (Mittler *et al.*, 2004). However, of the 75 ROS network genes with sufficient hybridization signal intensity to be detected on our microarray, a majority of the responsive transcripts were down-regulated >2-fold by NaCl (Table 2-2). The predominance of non-responsive or down-regulated transcripts was evident within almost every category of ROS-scavenging enzymes, except foralternative oxidase, ferritin, and monodehydroascorbate reductase, in which all NaCl-responsive transcripts were induced. The limited proportion of genes with NaCl-inducible transcripts within the ROS-scavenging network points to a considerable amount of sub-functionalization at the regulatory and catalytic levels, as well as roles for these genes beyond detoxification of ROS (Noctor, 2006). A third family of peroxidases, namely the type III (heme-containing) peroxidases, is not usually considered part of the ROS scavenging network and may have diverse roles including generating ROS for cell wall modification (Passardi *et al.*, 2005). We arbitrarily selected three peroxidase genes PER21 (At2g37130), PER69 (At5g64100), and PER27 (At3g01190) for validation by qRT-PCR. The results confirmed that these genes were generally down-regulated by NaCl-treatment (Table 2-1). These observations are consistent with functions for type III peroxidases in biological processes that are largely distinct from ROS scavenging.

A large proportion of glutathione-S-transferases (GSTs) were induced by NaCl treatment (Table 2-2). We note that nearly all of the NaCl inducible GSTs are from the Tau family of GSTs, highlighting a distinct role for this sub-class of genes in oxidative stress responses, as has been previously inferred from proteomic studies (Kilili *et al.*, 2004).

Transporters

The regulated transport of molecules across the plasma and vacuolar membranes is a well characterized response to abiotic stress (Sahi *et al.*, 2006). Within the NaCl-responsive transcripts on our microarray, we observed enrichment of transporters for water, sugars, cations, and other molecules (Table 2-2). The NaCl-responsive aquaporins and vacuolar-type ATPases detected on our microarray were enriched almost exclusively in down-regulated transcripts. In contrast, almost all of the detectable NaCl- responsive Na^+/H^+ exchangers were induced by stress. The homogeneity of these responses indicates relatively little sub-functionalization within these groups of genes, as compared to the ROS scavenging network described above. Among the remaining antiporters, as well as sugar transporters, ABC (ATP-binding cassette), and LeOPT1-like transporters and MATE-like (multi-antimicrobial extrusion) efflux carriers, specific transcripts were induced and others were repressed by NaCl-treatment (Table 2-2) (Ward, 2001; Rogers and Guerinot, 2002). Within the MATE family in particular, the proportion of NaCl-inducible transcripts is relatively large, as transcripts representing approximately half of the 26 detectable genes were induced >2 fold by NaCl, while only one MATE gene was equivalently repressed. One of these transcripts was among those we selected for further validation by qRT-PCR (Table 2-1). Both qRT-PCR and microarray analysis showed an approximately 8-fold increase in transcript abundance after 6h of NaCl treatment, with further accumulation to a 32-fold increase by 48h. The large proportion of induced genes within the MATE family, plus the magnitude of their induction, suggests a previously underappreciated role for MATE-efflux carriers in the root response to NaCl. Very little is known about this family of carriers, although a previous meta-analysis of microarray data identified some MATE transporters among a list of stress-induced transporters (Maathuis, 2006).

Primary energy metabolism, carbohydrates and cell walls

Transcripts required for the main respiratory pathways of glycolysis, the tricarboxylic (TCA) cycle, and the pentose phosphate pathway (PPP) were generally non-responsive or were down-regulated by NaCl treatment (Table 2-3). Genes encoding components of the mitochondrial electron transport chain were especially enriched among down-regulated profiles (Table 2-3). The down-regulation of these energy-evolving

Table 2-3. NaCl-responsive transcripts related to respiration and carbohydrate and cell wall metabolism.

family, group, or pathway	number of genes in genome	on array	signal above bkgnd.	>2 fold up	>2 fold down	assignment to STEM profiles (Fig. 2-3) cluster:# of genes
glycolysis*	70	64	45	6	10	a:2;b:1;d:1;f:1;h:2; i:3;j:5;k:3
tricarboxylic acid cycle	56	52	34	6	11	a:4;b:1;f:1;g:1; i:4;j:7
mitochondrial respiration*	95	66	55	0	26	g:1;i:2;j:23;o:1
non-oxidative pentose phosphate pathway	13	12	8	1	6	h:1;i:1;j:3;k:1;o:1
oxidative * pentose phosphate pathway	19	19	13	0	5	i:3;j:1;k:1
glycosyltransferase*	279	269	113	29	26	a:12;b:8;c:2;d:4;f:3; g:2;h:1;i:10;j:10;k:1; l:1;n:1;o:3
glycoside hydrolase (GH)*	306	289	121	42	27	a:17;b:16;c:1;d:4;e:3; h:2;i:11;j:6;k:2;l:2;o:3 p:1
GH family 16*	27	26	14	10	2	a:4;b:4;d:1;e:1;j:1; o:1
GH family 28*	52	46	10	4	1	a:3;b:1;d:1
expansins	35	32	21	7	4	a:1;b:3;c:1;d:2;i:3; m:1
lignification (exclusive of 4-CL like and COMT-like)	39	36	20	3	7	a:1;c:1;d:1;i:1;j:5; o:1
4CL-like*	9	8	3	2	1	b:1;d:1;k:1
COMT-like*	13	12	5	5	0	a:1;b:3
lipid transfer protein*	70	64	31	5	15	a:3;b:1;h:1;i:1;j:8; k:2;n:2;o:2

Groups of genes that are significantly enriched in at least one STEM profile at a FDR of <5% are marked with an asterisk (*). The final column details the frequency of assignment of individual genes to specific STEM profiles identified by corresponding letters in Fig 2-3. Functional categories in this table are defined according to the following sources: glycolysis, TCA, electron transport and PPP (Zhang *et al.*, 2005); all other groups: (Rhee *et al.*, 2003).

pathways is a common stress response that may serve to conserve energy and limit growth and further generation of ROS (Moller, 2001). The few glycolytic and TCA-related transcripts that were up-regulated were all isoforms of other genes that had been

down-regulated, although no correlation between cytoplasmic location and NaCl-response could be detected (data not shown). Transcript abundance for almost all components of the PPP was also decreased (Table 2-3), including D-ribulose-5-phosphate 3-epimerase (At3g01850, At1g63290), ribose 5-phosphate isomerase (At2g01290, At3g04790), 6-phosphogluconolactonase (At5g24410, At3g02360), and glucose-6-phosphate-1-dehydrogenase (G6PDH). The PPP is an important source of NADPH, especially in non-photosynthetic tissues, and therefore might be expected to have increased activity under oxidative stress (Rizhsky *et al.*, 2002; Chen *et al.*, 2004; Saher *et al.*, 2005). However, we noted that the detected down-regulated G6PDH transcripts were G6PD5 (At3g27300) and G6PD6 (At5g40760), which are the major cytosolic versions of G6PDH expressed in Arabidopsis roots and are distinct from the four genes encoding plastid-localized isozymes (Wakao and Benning, 2005). Previous analyses of G6PDH enzyme activity in potato and tobacco leaves has shown that isozymes located in plastids, but not the cytosol, have increased enzymatic activity following exposure to osmotic and oxidative stress (Hauschild and von Schaewen, 2003; Debnam *et al.*, 2004). Thus, G6PD5 and G6PD6 may function preferentially under conditions other than osmotic and oxidative stress. The sub-functionalization of isozymes into groups with contrasting responses to stress appears to be a common theme within the PPP, TCA, and glycolysis-related genes detected on our array.

Glycosyltransferases (GTs) and glycoside hydrolases (GHs) are two large superfamilies of carbohydrate- active enzymes (Coutinho and Henrissat, 1999). All GTs catalyze similar biochemical reactions, namely the transfer of sugar moieties to acceptor molecules. Conversely, GHs break bonds existing between sugar moieties and other molecules. Within each of these superfamilies, a significant and roughly equivalent number of genes were either up-regulated or down-regulated by NaCl at the transcript level, suggesting dynamic regulation of glycosylation in response to stress. These salinity responsive genes were distributed throughout the many constituent gene families of GTs and GHs, such that only two of the constituent families of GHs were significantly enriched in NaCl-responsive genes by our criteria: GH16, and GH28 (Table 2-3.). One member of the GH16 family, *xyloglucan endotransglucosylase /hydrolase18* (XTH18; At4g30280), was previously reported to be expressed in the

base of elongating roots, and is among the NaCl-induced genes we selected for validation by qRT-PCR (Table 2-1) (Vissenberg *et al.*, 2005). GH16 and GH28 are comprised largely of xyloglucanases and galacturonases, respectively, which are both often associated with cell wall metabolism. A limited number of other cell-wall related gene families were also enriched in NaCl-responsive transcripts, most notably the expansins (Table 2-3) (Lee *et al.*, 2001), for which at least one-third of the genes were transcriptionally up-regulated by NaCl. Increased activity of expansins and xyloglucanases is consistent with the increase in cell wall flexibility observed in response to osmotic stress in some species (Jones and McQueen-Mason, 2004).

A decrease in transcript abundance for many enzymes involved in lignification, as well as the potentially cell wall-related lipid transfer proteins (LTPs), were also observed from the microarray data (Table 2-3). However, we note that two families of enzymes that are sometimes grouped in the "lignification tool box", namely the 4CL (4-coumarate:CoA ligase)-like and the COMT (Caffeic acid O-methyltransferase)-like families (Raes *et al.*, 2003), were almost entirely up-regulated in response to NaCl stress. It is should be emphasized that these two families do not include their namesakes *4CL* and *COMT*, which are enzymes with clearly established roles in lignification. Thus the 4CL-like and COMT-like genes may have unique roles in the NaCl stress-response, and may not necessarily be related to lignification. LTPs are another group of proteins, some of which have been shown to affect cell wall extensibility or to be secreted in response to NaCl stress (Dani *et al.*, 2005; Nieuwland *et al.*, 2005). Fifteen LTP transcripts on our microarray were down-regulated by NaCl treatment (Table 2-3), although 5 LTPs were up-regulated. It is likely that these various LTPs represent a diversity of stress responses, some of which may also be related to cell-wall extensibility.

Protein metabolism

The impact of abiotic stress on protein metabolism is evident in our microarray results. A decrease in bulk protein translation following NaCl has been detected in proteomic studies, and is consistent with our observed down-regulation of the majority of transcripts for almost all cytosolic and plastidic ribosomal proteins (Table 2-4) (Ndimba *et al.*, 2005). Among proteins that promote the proper folding of proteins

and/or prevent the aggregation of nascent or damaged proteins, we detected enrichment of NaCl-responsive genes within several gene families. Within the peptidyl prolyl isomerase super-family, the 29 genes represented by detectable transcripts were largely down-regulated by NaCl (Table 2-4) (He *et al.*, 2004), although only the cyclophilins were significantly (<5% FDR) enriched in down-regulated transcripts. A second superfamily of genes related to protein folding and aggregation are the heat shock proteins (HSPs) (Lin *et al.*, 2001; Larkindale *et al.*, 2005). As expected, a majority of the 51 detectable HSP superfamily genes were transcriptionally induced by NaCl, and transcripts for only 5 genes were down-regulated by >2 fold (Table 2-4). Although the majority of HSPs are resident in the cytosol, most of the 5 down-regulated genes detected in the HSP superfamily were targeted to specific compartments, including two genes targeted to the endoplasmic reticulum (ER) (HSP70, At4g16660; HSP90-like *SHEPERD*, At4g24190), a chloroplast targeted HSP100/CLP (At5g50780). Consistent with these observations, some HSP family proteins that are targeted to chloroplasts or the ER have been previously shown to be repressed by particular abiotic stresses (Sung *et al.*, 2001).

Proteolytic activity has been attributed to over 600 peptidases within the plant genome, (Rawlings *et al.*, 2004). From our microarray data, both up- and down-regulated transcripts were detected for genes within each of the aspartic, cysteine, serine, and metallo-peptidase super-families (Table 2-4). Specific families and sub-families within the cysteine and serine peptidases were significantly enriched in NaCl responsive genes, as shown in Table 2-4. In contrast to the other peptidase superfamilies, the responsive threonine peptidases that comprise the 20S catalytic core of the 26S proteasome were exclusively down-regulated. A predominance of down-regulation was also observed among the transcripts comprising the other component of the 26S proteasome, namely the 19S regulatory particle (Table 2-4). However, many of the gene families putatively encoding functions upstream of the 26S proteasome in the ubiquitination pathway were up-regulated. Notably, SKP1 (S-phase-associated kinase protein) kinases, E3 U-box proteins, and E3 RING (Really Interesting New Gene) finger proteins were all significantly enriched in NaCl-induced genes (Table 2-4). Because the 26S proteasome works in conjunction with the ubiquitination pathway as

Table 2-4. NaCl-responsive transcripts related to protein metabolism.

family, group, or pathway	number of genes in genome	on array	signal above bkgnd.	>2 fold up	>2 fold down	assignment to STEM profiles (Fig. 2-3) cluster:# of genes
ribosome large subunit*	121	100	83	0	59	i:19;j:36;n:4
ribosome small subunit*	94	87	74	0	53	i:24;j:24;n:1;o:1
ribosome plastidic	13	13	9	0	3	i:1;j:1;n:1
peptidylprolyl isomerase (PPI)						
PPI: cyclophilin*	28	28	19	1	9	b:1;d:1;i:1;j:8;l:1; m:1
PPI: FKB	23	22	7	1	2	a:1;j:2
PPI: parvulin	3	3	3	0	2	i:1;j:1
heat shock proteins*	70	68	51	36	5	a:31;b:3;c:1;f:1;i:2; j:2;o:1
peptidase*	557	524	265	43	73	a:20;b:12;c:1;d:4;f:6; g:4;h:1;i:16;j:46;k:3; l:2;n:2;o:6
aspartic peptidase	76	69	31	7	8	a:4;b:2;f:1;i:2;j:5;o:1
cysteine peptidase	102	102	55	11	15	a:5;b:2;d:3;f:3;i:4; j:7;k:2;l:1;n:1;o:1
family C26*	10	10	9	4	2	a:1;b:1;d:3;i:1;j:1
metallopeptidase	85	78	44	4	6	a:2;f:2;i:1;j:4
serine peptidase*	261	244	112	18	31	a:8;b:6;c:1;d:1;g:4; h:1;i:8;j:18;k:1;l:1; n:1;o:4
sub-family S10-004*	19	17	7	0	6	i:1;j:5
sub-family S10-005*	24	24	9	0	3	g:1;j:1;o:2
sub-family S33-UPW*	45	42	22	6	7	a:2;b:2;c:1;d:1;g:2; i:1;j:3;l:1;o:1
threonine peptidase *	28	28	22	3	13	a:1;b:2;i:1;j:12
20S proteasome subunit*	19	19	17	0	12	i:1;j:11
19S proteasome*	33	31	24	0	10	i:3;j:6
SKP1/ASK1*	17	16	9	5	3	a:2;b:1;h:2;j:3
E3 RING-type*	430	414	178	47	34	a:16;b:17;c:4;d:8;f:4; h:3;i:14;j:14;k:1;l:3;n:1 p:2
E3 Ubox-type*	61	53	30	16	3	a:6;b:8;c:1;d:1;j:2; o:1
Ubox class III*	12	11	9	7	2	b:5;c:1;d:1;j:1;o:1

Groups of genes that are significantly enriched in at least one STEM profile at a FDR of <5% are marked with an asterisk (*). The final column details the frequency of assignment of individual genes to specific STEM profiles identified by corresponding letters in Fig 2-3. Functional categories in this table are defined according to the following sources: ribosomes (Barakat *et al.*, 2001) ; PPIs (He *et al.*, 2004); peptidases (Rawlings *et al.*, 2004); 19S

proteasome (Kanehisa and Goto, 2000); E3 RING (Stone *et al.*, 2005); HSPs, SKP1s, E3 Ubox (Rhee *et al.*, 2003).

the major proteolytic system of the nucleus and cytosol, it appears paradoxical that many ubiquitination-related proteins would be up-regulated, while the detectable 26S proteasome transcripts would be uniformly down-regulated by NaCl treatment (Smalle and Vierstra, 2004). This suggests a role for some SKP1, E3 U-box proteins, and E3 RING related enzymes that is independent of the 26S proteasome in the stress response, and may also hint at aspects of regulatory complexity not captured by microarray analysis (Kawaguchi *et al.*, 2004).

Signal transduction components and hormones

The predicted kinases of Arabidopsis form a very large superfamily of at least 979 genes that has been subdivided into 5 classes comprised in total of 81 families (Tchieu *et al.*, 2003; Shiu *et al.*, 2004). Nearly all of the NaCl-responsive kinases detected from our microarray were associated with the two largest classes in the PlantsP classification system (PPC:1, PPC:4) and twelve families therein (Table 2-5). These include eight families of receptor-like kinases (RLKs) and related kinases: legume lectin domain (LLD), leucine rich receptor (LRR) II & X, and LRR XI &XII, receptor like cytoplasmic kinase (RLCK) VII, wall-associated kinase (WAK)-like, proline-rich extensin-like kinases (PERK), S-Domain (type 1), and domain of unknown function (DUF) 26. Within each of these families, nearly all NaCl-responsive genes increased in transcript abundance following stress-treatment, and none of the detectable PERKs or S-Domain (type 1) kinases was repressed by NaCl (Table 2-5). Aside from the RLKs and related proteins in class PPC:1, we also detected enrichment of NaCl-responsive transcripts within several families of non-transmembrane kinases, including Mitogen Activated Protein Kinase Kinase Kinases (MAP3Ks), Calcium Responsive Kinases (CRKs), Ribosomal Protein S6 Kinases (RPS6K), and an unnamed family (PPC:4.4.1) of kinases. Among these, up-regulated transcripts were especially predominant in the RPS6K and PPC:4.4.1 families.

Protein phosphatases are divided into three major classes: Protein Serine /Threonine Phosphatases (STKs), Dual Specific Phosphatases (DSPs), and Protein

Table 2-5. NaCl-responsive transcripts related to signal transduction and hormone biosynthesis.

family, group, or pathway	number of genes: in genome	on array	signal above bkgnd.	>2 fold up	>2 fold down	assignment to STEM profiles (Fig. 2) cluster:# of genes
kinases	979	921	426	164	66	n,o:1;a:61;b:69;c:11;d:7; e:1;f:10;g:2;h:4;i:16;j:30 k:3;l:1;n:1;o:12;p:1
PPC:1 *	564	530	238	105	38	a:36;b:49;c:6;d:5;e:1; f:3;g:2;h:3;i:6;j:17;k:1; l:1;n:1;o:10
1.11.1 LLDK*	41	40	22	13	3	a:8;b:4;g:1;j:1;o:2
1.12.2 LRR II & X*	18	17	10	4	0	a:1;b:3
1.12.4 LRR XI & XII	45	44	27	8	5	a:2;b:2;c:1;d:2;f:1; h:1;i:1;j:3;n:1
1.2.2 RLCK VII*	45	44	25	19	0	a:6;b:9;c:1;d:2;e:1;f:1
1.5.1 WAK	27	23	11	6	5	b:3;c:1;j:3;l:1;o:1
1.6.2 PERK*	18	17	6	4	0	a:1;b:3
1.7.1 S-Domain (type 1)	14	14	10	7	0	a:3;b:3;c:1
1.7.2 DUF 26*	66	61	29	17	5	a:5;b:11;f:1;i:1;j:2;o:1
PPC:2	52	51	22	6	6	a:3;b:2;f:1;i:2;j:2;k: 1;o:1
PPC:4 *	279	265	133	47	18	n,o:1;a:20;b:18;c:5; d:1;f:5;i:6;j:10;o:1
4.1 MAP3K*	51	48	20	10	2	a:6;b:3;d:1;i:1
4.2 CRK*	123	119	58	21	10	n,o:1;a:5;b:10;c:3; f:3;i:3;j:4;o:1
4.2.6 RPS6K *	40	40	16	7	1	n,o:1;b:6;c:1;f:1
4.4 unclassified*	27	23	8	7	1	a:1;b:4;c:1;f:1;j:1
phosphatase*	125	116	64	24	9	a:9;b:11;c:3;d:1;g:1; i:5;j:1;k:1
PPC:6.1 (STKs)	23	19	12	0	3	i:2
PPC:6.2 DSP	8	8	6	1	2	a:1;i:1;j:1
PPC:6.3 PP2C *	64	62	38	22	3	a:7;b:11;c:3;g:1;i:1;k:1
response regulator: A *	11	11	8	4	1	a:1;b:1;c:2;i:1
similar to MLO proteins*	14	14	5	3	2	b:3;i:1;j:1
14-3-3 proteins*	13	13	8	0	5	i:1;j:4
ethylene biosynthesis	28	27	16	6	5	a:2;b:3;f:1;i:2;j:2
jasmonic acid biosynthesis*	20	19	11	7	3	a:1;b:5;d:1;j:2;o:1
IAA biosynthesis*	10	9	7	2	2	a:1;f:1;h:1;i:1;n:1
auxin transport*	8	8	4	3	1	a:2;b:1;i:1
Lateral Organ Boundaries class I*	36	34	11	7	0	a:2;b:1;c:3;g:1;p:1

Groups of genes that are significantly enriched in at least one STEM profile at a FDR of <5% are marked with an asterisk (*). The final column details the frequency of assignment of individual genes to specific STEM profiles identified by corresponding letters in Fig 2-3.

Functional categories in this table are defined according to the following sources: kinases and phosphatases (Tchieu *et al.*, 2003); hormone biosynthesis (Zhang *et al.*, 2005); all others: (Ward, 2001; Rhee *et al.*, 2003).

Phosphatase 2C (PP2C) (Tchieu *et al.*, 2003). We observed that the STKs and DSPs differed greatly from the PP2Cs in their transcriptomic response to NaCl-shock (Table 2-5). Transcripts for only one of the 18 detected STKs or DSPs were strongly induced by NaCl treatment, whereas transcripts for more than half of the detectable PP2Cs were strongly induced by NaCl. Some PP2Cs have been previously identified as components of the ABA signalling pathway, and all of these are among the NaCl-induced PP2Cs detected from our arrays: *ABI1* (*ABA INSENSITIVE 1*; At4g26080), *ABI2* (At5g57050), *HAB1* (*HOMOLOGY TO ABI1/ABI2*; (At1g72770), *HAB2* (At1g17550), and *AHG3* (*ABA-HYPERSENSITIVE GERMINATION3*, At3g11410) (Leung *et al.*, 1997; Merlot *et al.*, 2001; Saez *et al.*, 2004; Yoshida *et al.*, 2006). Our data indicate that nearly twenty other PP2Cs, including those outside of the ABI/HAB clade defined by Saez and colleagues, may play important roles in NaCl stress signalling.

The two-component signal transduction system, which consists of histidine protein kinases (HKs), His-containing phosphotransfer proteins (HPs) and response regulators (RRs), is involved in plant hormone, stress, and light signaling (Hwang *et al.*, 2002). The Arabidopsis genome contains 54 genes encoding putative HKs, HPs, RRs, and related proteins. In our data, none of the 16 HKs were induced by salt, while *AHK1*, a putative osmosensor (Urao *et al.*, 1999), was down-regulated at the 6h time-point. However, the A-type RRs were significantly enriched among clusters of NaCl-induced genes (Table 2-5). These include *ARR5*, *ARR6*, and *ARR7*, which act as negative regulators to repress the expression of the cytokinin-responsive genes (Hwang and Sheen, 2001), suggesting a potential link between NaCl stress and cytokinin signalling.

A handful of other families of putative regulatory proteins were statistically enriched among clusters of NaCl-responsive genes in our analysis. Transcripts for at least half of the six MLO (mildew resistance locus o) proteins detected on our microarray were induced by NaCl-treatment (Devoto *et al.*, 2003). These are seven-transmembrane domain proteins with features similar to G-protein coupled receptors,

and some members of this gene family have been reported to be abiotic stress responsive (Chen *et al.*, 2006). Finally, transcripts decreased for at least 5 of the 8 14-3-3 genes detectable in our root microarray, while no significant increase in transcript abundance for any 14-3-3 protein genes was observed (DeLille *et al.*, 2001).

Ethylene and jasmonic acid (JA) are hormones whose activity has been previously correlated with environmental stress (Devoto and Turner, 2005; He *et al.*, 2005; Ludwig *et al.*, 2005). The rate-limiting step of ethylene biosynthesis is the production of 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), which is followed by the conversion of ACC to ethylene by ACC oxidase (ACO) (Chen *et al.*, 2005). ACS and ACO are each encoded by gene families. Four ACC synthase and one ACC oxidase genes were significantly up-regulated by NaCl-treatment, while another 3 ACO genes were down-regulated by salt. Differential regulation of members of the ACO gene family indicates that although ACS is the key regulatory point for ethylene biosynthesis, regulation of ACO expression also has functional significance (Chen *et al.*, 2005). Ethylene signalling transduction involves ethylene response factor (ERF)/ ethylene response element binding protein (EREBP), which have been shown to act as activators or repressors of additional downstream ethylene-responsive genes. JA and its derivatives are also known to be involved in a wide range of developmental processes, as well as stress and defence responses (Devoto and Turner, 2005). At least 7 genes implicated in the biosynthesis of JA were induced by salt stress (Table 2-5), including *LIPOXYGENASE3* (*LOX3*, At1g17420), *ALLENE OXIDE CYCLASE* (*AOC1*, At3g25760), *AOC2* (At3g25780), and *ALLENE OXIDE SYNTHASE* (AOS, At5g42650), indicating that biosynthesis of this hormone is also NaCl responsive.

Within the biosynthetic pathway for indole acetic acid (IAA), which is an auxin, we detected up-regulation of two genes in response to NaCl treatment: namely nitrilases *NIT1* (At3g44310) and *NIT2* (At3g44300) (Table 2-5) (Woodward and Bartel, 2005). Consistent with an increase in levels of bioactive auxin, we also detected down-regulation of transcripts for *SUPERROOT2* (*SUR2*, At4g31500), an auxin conjugating enzyme (Barlier *et al.*, 2000). We confirmed the repression of *SUR2* transcripts by qRT-PCR (Table 2-1). Furthermore, we observed that of 4 putative auxin efflux carriers detectable after hybridization, transcripts for *PIN1* (At1g73590), *PIN3*

(At1g70940), and *PIN7* (At1g23080) were induced by our NaCl treatment. Part of the increased auxin activity we inferred from our microarray data may be directed towards increased lateral root formation, which is an auxin-regulated, and PIN requiring process that has been previously described in NaCl-treated roots (He *et al.*, 2005; De Smet *et al.*, 2006). Lateral root formation also involves genes from the *LATERAL ORGAN BOUNDARIES* (*LOB*) family; consistent with this requirement, we observed that the class I sub-group of this family was exclusively enriched in up-regulated transcripts, with at least seven genes induced following NaCl-treatment (Table 2-5)(Shuai *et al.*, 2002). Thus, the initiation of lateral root primordia is a NaCl-stress response that appears to be distinctly represented within our microarray data, even though the process involves only a small number of cells within the entire root.

Transcription Factors

The Arabidopsis genome encodes about 1,922 predicted transcription factors (TFs), which have been classified into 64 families (Riechmann *et al.*, 2000; Guo *et al.*, 2005; Iida *et al.*, 2005). From the over 1,613 transcription factors represented as probes on our microarray, transcripts for 734 genes were detected at a signal intensity above background. Of these, 289 predicted TFs were up-regulated and 139 transcription factors were down-regulated at least 2 fold at one or more time points following NaCl treatment (Table 2-6). STEM analysis identified 17 families that were significantly enriched among clusters of NaCl responsive genes. These TF families range in size from 7 to 203 members, and are described below. Although these 17 families contain almost two-thirds of the predicted TFs in the Arabidopsis genome, it is worth noting that transcripts for several other relatively large TF families were detected on the array, but these were largely unresponsive to NaCl stress. Some of the notable TF families that were expressed in roots, but which were not significantly enriched in NaCl-responsive transcripts are: MADS (MCM-1, Agamous, Deficiens and Serum Response Factor), ARF (Auxin Response Factor), Nin-like (nodule inception), and TUB (Tubby).

At least four TF families have been reported to be enriched in stress-responsive genes: MYB (Myeloblastosis), HSF (Heat Shock Factor), AP2/EREBP (Apetala-2/EREBP), and WRKY (named after the WRKY amino acid motif). A recent analysis of MYB-transcription factor gene expression concluded that almost all MYB TFs are

Table 2-6. Classification of NaCl-regulated transcription factors.

family, group, or pathway	number of genes in genome	on array	signal above bkgnd.	>2 fold up	>2 fold down	assignment to STEM profiles (Fig. 2) cluster:# of genes
ALFIN*	7	7	4	0	3	i:3
AP2/EREBP*	146	138	76	50	6	a:8;b:31;c:6;d:3;e:1; f:2;h:1;i:1;j:1;k:1;l:1; o:2
ARF*	23	20	12	4	0	a:3;d:1;f:1
AS2*	42	39	15	7	3	a:2;b:1;c:3;g:1;i:2;j:1; p:1
AUX/IAA	29	28	15	7	1	a:4;b:3;d:1;f:1;i:1;j:1
B3	39	36	8	2	2	a:1;b:1;i:2
bHLH	162	148	62	15	14	a:8;b:4;c:3;d:1;h:1;i:7; j:4;k:2;o:1;p:1
bZIP	75	68	42	11	11	a:5;b:5;c:2;i:3;j:4;l:1; o:2;p:1
C2C2-co-like*	31	31	13	5	2	b:1;d:1;e:1;f:2;i:1;j:1
C2C2-DOF*	36	31	15	8	1	b:5;c:1;d:2;g:1;k:1
C2C2-GATA*	29	27	14	3	3	b:3;d:2;h:1;i:1;j:1
C2H2*	130	122	57	22	14	a:5;b:15;c:1;d:1;f:1; i:5;j:6;o:2
C3H	33	31	13	5	2	a:1;b:3;c:1;j:1;n:1
CCAAT-HAP2	10	10	5	3	0	b:2;l:1
CCAAT-HAP3	11	11	7	2	2	a:2;i:1;j:1
CCAAT-HAP5	13	12	5	3	1	a:1;c:1;f:1;o:1
GARP-ARR-BARR19	12	12	4	1	1	b:1
GARP-G2-like*	43	43	26	8	8	b:4;c:1;d:3;f:1;i:3; j:4
GeBP	16	14	4	0	0	n/a
GRAS*	32	30	14	4	5	b:1;c:1;d:1;i:2;j:2;p:1
HB*	94	92	40	15	3	a:3;b:9;d:1;e:1;f:2;i:1; j:1;l:2;m:1
HMG	10	8	5	0	4	i:1;j:2;n:1
HSF*	24	23	13	10	1	a:6;b:4;d:1
JUMONJI	13	13	4	2	0	a:1;b:1;d:1
LIM	6	6	4	0	3	h:1;j:2;o:1
MADS	107	96	17	2	7	a:1;f:1;i:2;j:3;o:1
MYB*	203	188	84	33	12	a:9;b:18;c:1;d:5;f:2;i:3; j:5;n:1;o:1
NAC*	113	107	47	26	7	a:9;b:10;c:1;d:1;e:1;f:2; g:1;j:4;o:2
Nin-like	14	14	6	0	2	j:2
PHD	11	10	4	1	1	b:1;i:1
PLATZ*	9	8	5	2	0	b:2
SBP	16	15	8	3	1	a:1;b:2;i:1
TAZ	9	9	4	2	0	b:1;d:1;f:1
TCP	24	23	5	0	0	d:1

Trihelix	28	28	15	3	5	a:1;b:2;i:3;j:1;n:1
TUB	11	10	6	1	2	a:1;i:2
WRKY*	72	65	35	18	8	a:4;b:10;c:2;d:2;i:4; j:2;l:1
ZF-HD	15	15	6	1	2	d:1;j:2
ZIM*	15	12	10	8	1	b:3;c:3;d:1;f:1;o:1

Groups of genes that are significantly enriched in at least one STEM profile at a FDR of <5% are marked with an asterisk (*). The final column details the frequency of assignment of individual genes to specific STEM profiles identified by corresponding letters in Fig 2-3. Functional categories in this table are defined according to the Database of Arabidopsis Transcription Factors (Guo *et al.*, 2005). The following small transcription factor families, which are each represented by probes for three or fewer genes on the microarray, are not listed in this table: ARID, BES1, C2C2-YABBY, CAMTA, CCAAT-DR1, CPP, E2F/DPE2FC, EIL, FHA, GIF, GRF, HRT-like, LFY, LUG, MBF1, NZZ, PBF-2-like(Whirly), PcG, S1Fa-like, SAP, SRS, ULT, VOZ.

responsive to stresses or hormones (Chen *et al.*, 2006). Consistent with this report, we observed that at least one third of the 84 detectable MYB TFs were induced by NaCl at the transcript abundance level, and only a small fraction were repressed (Table 2-6). *MYB15*(At3g23250), which is a R2R3-type MYB gene (Stracke *et al.*, 2001), was induced approximately 16-fold by NaCl at each of the three time points, and this was confirmed by qRT-PCR (Table 2-1). Similarly, most of the HSF TFs detected on our array were up-regulated by NaCl, as expected for this family of stress-inducible TFs (Busch *et al.*, 2005)(Table 2-6). The AP2/EREBP family includes some of the best characterized stress-responsive transcription factors, including the CBF (CRT/DRE binding factors) proteins (Ohmetakagi and Shinshi, 1995; Gilmour *et al.*, 1998). We observed NaCl-inducible transcript accumulation in more than half of the 76 AP2 /EREBP TFs detectable on our array (Table 2-6); and these up-regulated transcripts were detected in each of the AP2/EREBP family's subgroups (i.e. AP2, RAV, ERF I-X) (Nakano *et al.*, 2006). We arbitrarily selected one AP2/EREBP TF (At1g44830, TINY-related) for confirmation by qRT-PCR. As shown in Table 2-1, both microarray and qRT-PCR analyses were in agreement about the strong inducibility of transcripts of At1g44830 following NaCl-treatment. The majority of WRKY family TF genes are known to be responsive to biotic and/or abiotic stress, although most researches have

focused on the roles of these genes in pathogen responses (Seki *et al.*, 2002a; Dong *et al.*, 2003; Narusaka *et al.*, 2004; Xu *et al.*, 2006). We observed that 18 transcripts out of the 35 WRKY TFs increased by at least 1.5 fold in response to NaCl treatment. *WRKY17* (At2g24570), *WRKY25* (At2g30250) and *WRKY33* (At2g38470) were chosen for further qRT-PCR validation. Both microarray and qRT-PCR confirmed that *WRKY17* and *WRKY33* transcripts increased at least 14-fold following NaCl treatment, although *WRKY17* transcript abundance peaked at 6h and gradually diminished, whereas *WRKY33* abundance remained very high throughout the time points sampled. The transcript level of *WRKY25* also increased and peaked at 48 h with an induction of at least 22-fold. These observations emphasize a potentially important role for WRKY transcription factors in abiotic stress responses.

Many TFs contain zinc-finger motifs including all members of the following families: C2H2 (Cys2-His2), C2C2 (Cys2-His2), C3H (Cys3-His) LIM (Lin-11, Isl-1 and Mec-3), PHD (Plant Homeodomain), WRKY, ZF-HD (zinc finger homeodomain), and ZIM (Zinc-finger protein expressed in Inflorescence Meristem). Of these, we observed the WRKY (as described above), and C2C2, C2H2, and ZIM families were particularly enriched in NaCl-responsive transcripts. The C2C2 and C2H2 families are relatively large, and had significant numbers both of up-regulated and down-regulated genes. The smaller ZIM family was represented by 10 genes with detectable transcripts in our analysis; 8 of these genes were up-regulated by NaCl treatment. Knowledge about the biological role of ZIM TFs is very limited. Previous studies have shown a putative relationship of some ZIM family members with GA-independent cell elongation in shoot tissues (Nishii *et al.*, 2000; Shikata *et al.*, 2004). The large proportion of ZIM family members with NaCl-inducible transcripts makes this group of TFs another attractive target for further functional characterization. We assayed the expression pattern of one ZIM transcription factor (At3g17860) by qRT-PCR and confirmed that it is induced after 6h of NaCl, although the transcript abundance falls off rapidly after 24h and 48h (Table 2-1).

Four other large families of TFs, each with distinct DNA binding motifs, are: bHLH (basic helix-loop-helix), bZIP (basic leucine zipper), NAC (NAM, ATAF1,2, CUC2), and HB (homeobox) genes. All of these families contained NaCl-induced

genes, although only the HB and NAC families were significantly enriched in NaCl-responsive transcripts. We selected one bHLH TF (*bHLH92*, At5g43650) and one NAC TF (At4g01550) for further confirmation by qRT-PCR. The NAC domain TF was chosen because its expression peaked at later time points than most TFs; *bHLH092* was selected because it was among the mostly highly induced transcripts of any gene on our microarray. Furthermore, this bHLH is not represented among probes on Affymetrix arrays, and therefore would not be detected in some previous transcriptomic studies, although it was among the NaCl-inducible transcripts identified in a long-oligomer microarray analysis of whole Arabidopsis plants (Kamei *et al.*, 2005). Our qRT-PCR analysis showed a NaCl-dependent increase of up to 50-fold for the NAC TF (At4g01550) transcripts, and an increase for *bHLH92* of over several magnitudes (Table 2-1). Clearly, these two TFs, and many of the other NaCl-responsive TFs shown in Table 2-6 are potentially important regulators of the NaCl-stress response in Arabidopsis roots.

An interesting property of the groups of transcription factors described in Table 2-6 is that the majority of the families (34/39) contain members that are assigned to different expression profiles. In many cases, related transcription factors show very divergent expression patterns, with some members of a single gene family induced early in the stress response, while other members are induced only at subsequent time points. For example, while the majority of AP2/ERF family members have a peak relative expression ratio at 6h (Table 2-6, and Fig 2-3, STEM profile b), some AP2/ERF transcripts had peak expression ratios at 24h (profile f), or increased in abundance throughout the experiment until at least 48h (profile a), while other genes did not show a significant relative change in expression until 48h (profile h), or showed a more complex pattern of regulation (profiles c, d, e), or were predominantly down-regulated (profiles i, j, k, l, o). Similarly divergent patterns of transcriptional responses have been described previously within families of oxidative stress inducible genes (Kim *et al.*, 2005). The large number of up- or down- regulated transcription factor genes we detected, as well as the diversity of their temporal expression patterns, is consistent with the existence of a highly complex regulatory network underlying the physiological response to NaCl treatment.

Conclusions

In this study, we identified 2,367 salt upregulated and 2,913 downregulated genes in Arabidopsis using the QiagenOperon microarray. Thus, micorarray profiling of NaCl-treated Arabidopsis roots revealed dynamic changes in transcript abundance for ca. 22% of the genome for all the three time points examined, including hundreds of transcription factors, kinases/phosphatases, hormone-related genes, and effectors of homeostasis, all of which highlight the complexity of this stress response.

Many transcription factors were induced during salt stress. By contrast, fewer transcription factors were downregulated during salt stress. These results suggest that salt responses in plants are initiated mainly by transcriptional activation rather than repression of genes. The downregulation of other transcription factors later in salt stressed roots may be the result of early activation of transcription factors.

Many of the salt-regulated genes reported here are regulated not only by salt but also by other abiotic and biotic stresses and by ABA (Seki *et al.*, 2002a, Seki *et al.*, 2002b; Kreps *et al.* 2002; Fowler and Thomashow, 2002; Lee *et al.*, 2005). Different signal transduction pathways may crosstalk and converge in the activation of these stress genes.

The observations of the transcriptomic response to NaCl-treatment in Arabidopsis roots were consistent with the broad themes of abiotic stress responses previously reported in other systems (Sahi *et al.*, 2006). However, our functional enrichment analysis showed that a considerable amount of sub-functionalization has occurred within many gene families, demonstrating the complex relationship between stress and processes related to reactive oxygen species, cell wall growth, transport, signal transduction, hormone signalling, proteolysis and transcriptional regulation. Moreover, STEM enrichment analysis allowed us to detect previously underappreciated families within these broad categories that are strongly associated with stress response, including MATE transporters, LeOPT1-like transporters. PERK kinases, MLO-like receptors, carbohydrate active enzymes (e.g. XTH18), transcription factors, and other proteins (e.g. 4CL-like, COMT-like, LOB-Class 1). These data will facilitate the mapping of genetic regulatory networks in roots of Arabidopsis and a better understanding of stress phenomena in plants. The identified novel salt-responsive genes also

extend our ability to improve salt tolerance in plants. Further research could be directed towards exploring the network of gene regulation in the salt and determining the function of salt-responsive genes in salt tolerance through mutant analysis, overexpression, and other genetic and molecular biological approaches.

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Chapter 3: Comparative proteomic analysis of Arabidopsis root response to salt stress

Introduction

Soil salinity is a prevalent abiotic stress that limits the productivity and geographical distribution of plants. Roots are the site of perception and injury for several types of stress, including salinity, nutrient deficiency and heavy metals. Under many circumstan -ces, it is the stress-sensitivity of the root that limits the productivity of the entire plant (Atkin *et al.*, 1973; Steppuhn and Raney, 2005). An improved understanding of molecular mechanisms of responses in roots to NaCl treatment may therefore facilitate the development of crops with increased tolerance to NaCl and other stresses.

To build a useful description of the molecular mechanisms active in the response of roots to NaCl treatment, it is necessary to characterize the components of these mechanisms, including proteins. Proteomic profiles have been investigated for various stresses and species, including NaCl-treated roots of pea (*Pisum sativum* L.) (Kav *et al.*, 2004), rice (*Oryza sativa* L.) (Yan *et al.*, 2005) and wheat (*Triticum aestivum* L.) (Majoul *et al.*, 2000), drought treated rice (Salekdeh *et al.*, 2002), sugar beet (Hajheidari *et al.*, 2005), poplar (*Populus trichocarpa* Torr. & A.Gray) (Plomion *et al.*, 2006) and chickpea (*Cicer arietinum* L.) (Pandey *et al.*, 2007), and cold treated Arabidopsis (Bae *et al.*, 2003) and rice (Cui *et al.*, 2005), as well as heat treated rice (Lee *et al.*, 2007) and wheat (Majoul *et al.*, 2004). Recently, a 2-DGE based technology was employed to identify NaCl and osmotic responsive proteins in Arabidopsis cell suspension and root microsomal fraction (Ndimba *et al.*, 2005; Lee *et al.*, 2004). In the present study, we applied a moderate NaCl stress to hydroponic cultured Arabidopsis roots to identify proteins that are responsive to NaCl treatment.

Transcriptome profiling, a widely-used technique to identify NaCl-responsive genes, has contributed to our understanding of salinity stress in species including

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Arabidopsis and rice (Kawasaki *et al.*, 2001; Kreps *et al.*, 2002; Seki *et al.*, 2002; Rabbani *et al.*, 2003; Chao *et al.*, 2005; Jiang and Deyholos, 2006). However, transcriptome profiling has some limitations because mRNA levels are not always correlated to those of corresponding proteins, due in part to post-transcriptional regulation. Only poor or moderate correlation between changes in the levels of specific mRNAs and their corresponding proteins has been previously reported in studies involving yeast (*Saccharomyces cerevisiae*), animals, or Arabidopsis (Gygi *et al.*, 1999; Tian *et al.*, 2004; Mooney *et al.*, 2006). Furthermore, post-translational modifications (PTM), such as phosphorylation and glycosylation, can result in a dramatic increase in proteome complexity without a concomitant increase in gene expression (Rose *et al.*, 2004; Jensen 2004). These biological realities motivated us to perform an analysis of NaCl stress responses at the proteome level, and to compare these results to our previous, microarray-based studies of similarly treated tissues (Jiang and Deyholos, 2006).

Materials and methods

Plant materials and stress treatment

Arabidopsis thaliana plants (wild-type, ecotype Col-0) were cultured hydroponically as described previously (Jiang and Deyholos, 2006). At 20 days after stratification (DAS), the hydroponic solution was changed to fresh, 1 x MS Murashige and Skoog (MS) medium, either with or without 150 mM NaCl, and was maintained for 6 or 48 h, with roots harvested from control or NaCl-treated plants in parallel at each time point and flash frozen in liquid nitrogen and stored at -80 °C. Three biologically independent replicates were prepared at separate times.

Physiological analyses

For root elongation assays, 5 DAS, wild-type Arabidopsis seedlings grown vertically on ½ x MS medium supplemented with 1% sucrose and 0.8% Phytablend (Caisson Labs) were transferred onto ½ × MS plates supplemented with 1% sucrose and 0, 50, 100, 150, 200, or 250 mM NaCl in square petri dishes, with root lengths measured after 7 d. For ion concentration determination, hydroponic cultured Arabidopsis roots and shoots (20 DAS) were dried for 2 d in an oven at 65°C. 100-500 mg of dry root or leaf

samples was digested according to EPA 3050B method (http://www.epa.gov/SW-846/pdfs/3050b.pdf) with modifications. Na and K concentrations were determined by flame emission spectroscopy (AAnalyst700, PerkinElmer). For the relative electrolyte leakage (REL) assay, ~ 150 mg of hydroponic cultured 18-DAS old seedlings were rinsed with ddH_2O and placed in test tubes containing 10 ml ddH_2O and incubated at room temperature for 2 h with the electrical conductivity of the solution (C_1) measured using conductivity meter (Orion 115Aplus, ThermoElectron). Then, the tubes were boiled for 15 min and cooled to room temperature, and the electrical conductivity (C_2) was measured again. The relative electrolyte leakage was calculated by the formula $C_1/C_2 \times 100\%$ (Cao *et al.*, 2007). Figures were drawn using SigmaPlot (v8.02, Systat Software Inc.).

Protein extraction and quantification

Total protein extracts were prepared essentially according to the method described by Tsugita and Kamo (1999) with modifications. In brief, approximately 1 g of control and NaCl-treated roots were homogenized separately to a fine powder in liquid nitrogen, and were transferred into three 2 ml tubes. 1 ml of 10% (w/v) trichloroacetic acid (TCA)/0.07% DTT in acetone was added to each tube and incubated at −20 °C for 1 h. Afterwards, tubes were centrifuged at 18,000x g for 15 min and the supernatants were discarded. The pellets were washed by resuspension in ice-cold acetone containing 0.07% DTT and centrifuged as described above. This wash was repeated three times, with the pellets dried at room temperature in a SpeedVac for 15 min and resuspended in 300 µL of lysis buffer (30 mM Tris-HCl, 7 M urea, 2 M thiourea, 4% CHAPS, pH 8.5). The samples were mixed vigorously, incubated overnight at 4 °C and centrifuged at room temperature for 15 min at 18,000 g, with the supernatants collected into fresh tubes. The protein extracts were cleaned up using 2-D Cleanup kit (GE Healthcare), dissolved in 200 µL of Destreak rehydration buffer containing 2% pH3-10 immobilized pH gradient (IPG) buffer (GE Healthcare), and quantified by using 2-D Quant kit (GE Healthcare) using bovine serum albumin (BSA) as the standard.

IEF and SDS-PAGE

Two-dimensional electrophoresis of protein extracts was performed using a GE Healthcare 2-DGE system according to the manufacturer's manuals. Briefly, IPG strips

(pH 3–10 NL, 24 cm) were rehydrated in 450 µl of Destreak rehydration buffer (containing 2% pH 3-10 IPG buffer) overnight (~15 h). Isoelectric focusing (IEF) was performed using IGPhor Isoelectric focusing unit with 400 µg of protein samples loaded by cup-loading. The voltage and duration used were as follows: 1st step and hold: 300 V, 3 hours; 2nd gradient: 1000 V, 6 hours; 3rd gradient: 8000 V, 3 hours; 4th step and hold: 8000 V, 4 hours 40 minutes. Prior to second dimension separation, the strips were incubated first in an equilibration buffer (6 M urea, 30% v/v glycerol, and 2% SDS in 0.05 M Tris-HCl, pH 8.8) containing 15 mM DTT for 15 min, then in an equilibration buffer containing 2.5% iodoacetamide (GE Healthcare) for another 15 min, followed by brief equilibration in 1 x SDS-Tris-glycine running buffer for 5 min. The 2nd dimension separation of proteins were performed on SDS-PAGE gel (12.5% acrylamide, Bio-Rad) using the Ettan Dalt Six apparatus (GE Healthcare) with protein markers (Cat#SM0661, Fermentas) loaded at the left most side. The electrophoresis was carried out at ~25 ºC and 2.5 w/gel for 30 min and then 17 w/gel for ~5 h 40 min until the bromophenol blue dye front arrived at the bottom of the gels. Following SDS-PAGE, gels were washed in ddH_2O three times for 15 min and proteins were detected by a modified Collodial Coomassie Brilliant Blue staining-blue silver protocol, which was assessed to have the sensitivity comparable to silver staining (Candiano *et al.*, 2004). After three time washes in ddH_2O, the 2-D gels were scanned immediately using a Fuji FLA-5000 scanner (Fujifilm) with a resolution of 100 µm and 16-bit grayscale pixel depth. A total of 12 gels were analyzed: i.e. three gels (biologically independent replicates) for each of two treatments (0mM or 150mM NaCl) at each of two time points (6 or 48 h). This experimental design was balanced with respect to all conditions, and the use of three gels per condition is consistent with recommendations based on previous statistical analyses of protein gel electrophoresis (Hunt *et al.* 2005). For pI and MW calibrations, we used the 2-D internal standards (Cat#161-0320, Bio-Rad) with IEF, SDS-PAGE, staining and scanning performed the same as above.

Image and statistical analysis

2-D gel images were analyzed using ImageMaster 2-D Platinum 6.0 (GE Healthcare). To verify the autodetected results, all spots were manually inspected and edited as necessary. After spot detection, quantification, and background subtraction, each

analyzed gel was matched individually to the reference gel, and matched spots were grouped into subclasses. To compensate for subtle differences in sample loading, gel staining, and destaining, the volume of each spot (i.e. spot abundance) was normalized as relative volume. This normalization method, provided by ImageMaster 2D Platinum 6.0 software, divides each spot volume value by the sum of total spot volume values to obtain individual relative spot volumes. Class reports were generated for spots of interest. The differences in expression between control and treatment were analyzed by the Student's *t*-test with $P \leq 0.05$ considered significant. The molecular masses of proteins on gels were determined by co-electrophoresis of standard protein markers (Fermentas) and internal 2-D internal standards (Bio-Rad) according to the software manual. For the figures shown in the manuscript, spot IDs were renumbered using the annotation tool in ImageMaster Platinum 6.0 and image brightness and contrast was adjusted and the protein marker sizes were added using Photoshop CS (Adobe).

In-gel digestion and LC-MS/MS analysis

Protein spots showing at least 1.5-fold difference in abundance between control and treatment at one or both time-points with $P < 0.05$ were selected and excised manually into 1.5 ml microtubes. The selection of 1.5-fold change as an arbitrary threshold allowed us to focus on the most responsive proteins for subsequent characterization, and is consistent with thresholds used previously by ourselves and others in microarray studies (Jiang and Deyholos, 2006 and references therein). Gel piece treatment and in-gel digestion of protein spots were performed following Jensen *et al.* (1999) with modifications. Briefly, gel pieces were first washed with 150 µL of HPLC grade water (Fisher Scientific), dehydrated with 50 µL of 100% acetonitrile (ACN), then destained with 100 µL of 50 mM NH_4HCO_3/50% ACN for 2 h or longer until colorless. After dehydration with ACN again, gel pieces were reduced in 30 µL of 10 mM DTT/0.1 M NH_4HCO_3 at 56 ºC for 30 min, dehydrated, and alkylated in 30 µL of 55 mM iodoacetamide/0.1 M NH_4HCO_3 at room temperature for 20 min in the dark, followed by rinsing with 200 µL of 0.1 M NH_4HCO_3, dehydrated with ACN, dried in a SpeedVac for 10 min. Afterwards, 20 µL of trypsin solution (0.02 µg/µL Trypsin Gold (Promega) in 40 mM NH_4HCO_3/10% ACN) was added and incubated on ice for 1 h, then at 37 ºC overnight (~14 h). Finally, 3 µL of 2% formic acid (FA) was added to

stop the digestion reactions, and the supernatant was collected into fresh tubes, followed by two extractions of peptides with 15 µL of 50% ACN/0.1% FA with the collect (~50 µL) mixed well and stored in -20 °C before use.

We performed LC-MS/MS analysis of digested peptide mixtures using an Agilent 1100 LC/MSD Trap XCT (Agilent Technologies). Briefly, an autosampler was used to inject 20 µL of each tryptic digest onto the first of two C-18 columns. This short 5 µm-enrichment column, Zorbax 300SB-C18 5 µm 5 x 0.3 mm, served to trap and concentrate the samples. Next, the sample was eluted onto the next C-18 column (Zorbax 300SB-C18 5µm 150 x 0.3mm), which was used in conjunction with a solvent gradient to separate the peptides. The peptide-separation gradient started at 85% solvent A (0.1% FA in H_2O) and ended at 55% solvent B (0.1% FA, 5% H_2O in ACN) over a 42-minute span. This was followed by a 10-minute period of 90% solvent B to cleanse the columns before returning to 97% solvent A for the next sample. The ion trap mass spectrometer collected information by first running a MS 300-2200 m/z scan and followed that with a MS/MS analysis of the most intense ions. In addition to the most intense ion for each scan, the software was set to exclude this ion after two spectra and gather MS/MS information on the next most intense ion(s). Raw spectral data was processed into Mascot Generic File (.mgf) format using the default method in the ChemStation Data Analysis module. MS/MS ion search was performed using MASCOT (http://www.matrixscience.com) searching the NCBInr database and taking *Arabidopsis* as the taxonomy. The parameters for searching were: a MS/MS tolerance of ±0.8 Da, one missed cleavage site, enzyme of trypsin, fixed modifications of carbamidomethyl (C), peptide tolerance of ± 2 Da, peptide charge of 1+ 2+ 3+, monoisotopic and ESI-TRAP instrument. Only significant hits, as defined by the MASCOT probability analysis ($P < 0.05$), were accepted. In case multiple significant hits were found for a protein, only the highest scoring hit was listed in Table 3-1.

Results

Plant growth response to NaCl stress

Arabidopsis thaliana is a glycophyte and is sensitive to NaCl exposure. To find an appropriate concentration of NaCl to use for treatment of plants prior to proteomic

profiling, we performed a root elongation dose response assay. Our results demonstrated that 100 and 150 mM NaCl inhibited root elongation by 53% and 78% respectively (Fig. 3-1A). However, concentrations higher than 200 mM NaCl almost completely inhibited root growth and led to death of almost all seedlings. In a post-stress recovery assay, 20 DAS plants were treated by 150 mM NaCl for 6, 24 or 48 h, and were transferred into fresh media to recover for 1 week. Almost all of the NaCl-treated plants recovered and resumed normal growth (data not shown). We therefore selected 150 mM as our treatment concentration, because it induced visible signs of stress including retarded growth rate and loss of turgor. This concentration of NaCl has been used in several previous gene expression studies, because it induces a moderate stress response and is not acutely lethal (Jiang and Deyholos, 2006; Ma *et al.*, 2006). Higher concentrations of NaCl appear to cause plasmolysis and lethality (Munns 2005), although other previous Arabidopsis studies have used a NaCl treatment concentration of 250, 300 or even 600 mM (Ndimba *et al.*, 2005; Seki *et al.*, 2002).

Relative electrolyte leakage (REL) is an indicator of membrane damage caused by NaCl stress. We measured stress-induced changes in plants treated with 150 mM NaCl. As shown in Fig 3-1B, the REL of seedlings treated by 150 mM NaCl for 6h was 8.6%.

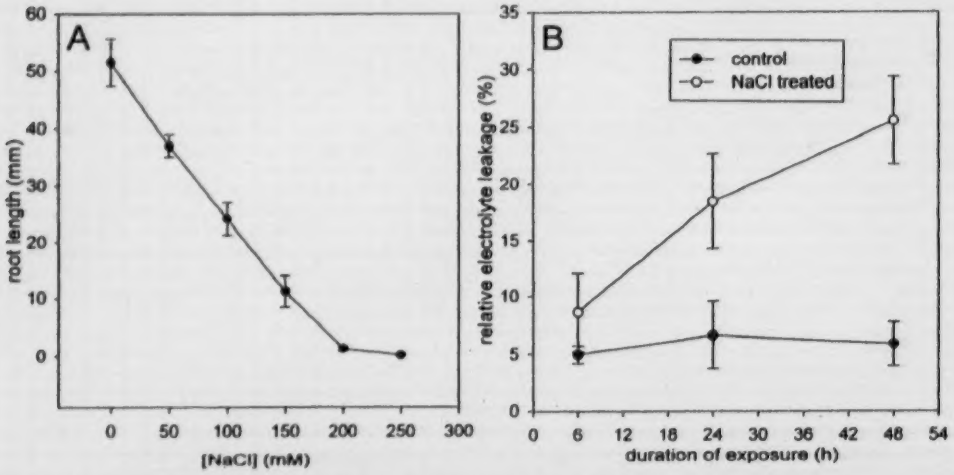

Fig. 3-1. Root elongation assay and relative electrolyte leakage. (A) Effect of increasing [NaCl] on root elongation of seedlings (n=9). (B) Relative electrolyte leakage from roots of control (filled circles) or NaCl-treated (closed circles) roots following 6, 24, or 48 h exposure to 150 mM NaCl (n=5). Vertical bars indicate standard deviation (S.D.).

After 24h treatment, the REL increased to 18% and further increased to be 26% after 48h of NaCl treatment. When compared to the control plants, the REL values of NaCl-treated seedlings were 1.7, 2.8 and 4.4 times higher at 6, 24 and 48 h, respectively, which indicated that stress-induced cellular damage accumulated throughout the duration of the experiment.

To further establish the physiological status of plants that we were to subject to proteome analysis, we next quantified the concentrations of K and Na in both leaves and roots of control and 150 mM-treated plants (Fig. 3-2). Na and K concentrations significantly increased and decreased, respectively, in both leaves and roots under 150 mM NaCl treatment. Moreover, the Na concentrations in roots treated by 150 mM NaCl for 6, 24, and 48h were 115, 167 and 132 fold higher than those of the control roots at comparable time points. The concentration of Na in roots reached a maximum after 24h (Fig. 3-2A), however, leaves continued to accumulate more Na after the 24h (Fig. 3-2B), presumably due to transpiration-driven flux. The K/Na ratios in both leaves and

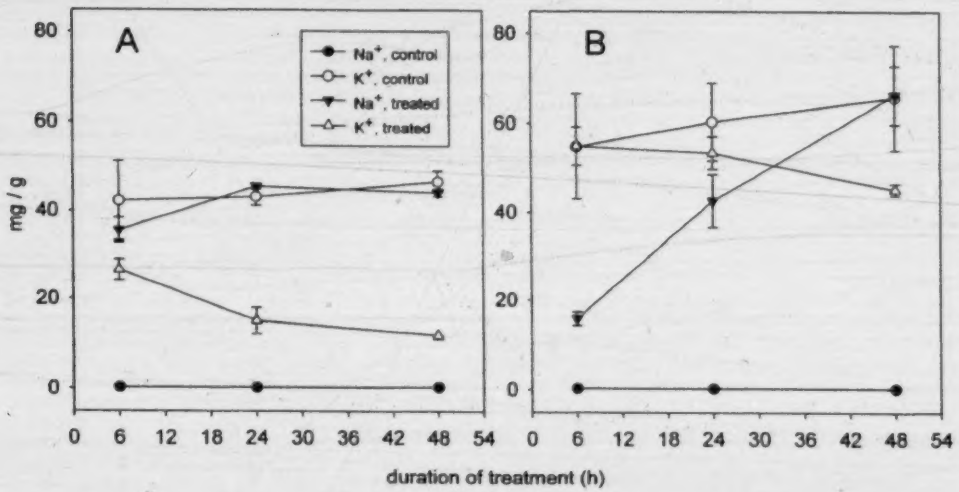

Fig. 3-2. Changes in Na and K ion concentrations following NaCl exposure. Na (filled symbols) and K (open symbols) concentrations (mg/g dry weight) were determined by flame emission spectroscopy in roots (A) or rosette leaves (B) of hydroponically grown Arabidopsis plants, following 6, 24, or 48 h of exposure to media supplemented with either 0 mM NaCl (control, circles) or 150 mM NaCl (treated, triangles). Na concentrations in control samples are very low (< 0.4 mg/g). Data are from 3 biological replicates, and vertical bars indicate ± S.D.

roots decreased as the NaCl treatment progressed. Part of the basis of Na^+ toxicity in plants is that high Na^+ concentration in the cells increases the Na/K ratio, which is adverse for most metabolic processes (Chinnusamy *et al.*, 2005). Thus, the quantitative ion analysis was consistent with the REL data in describing the progressive accumulation of stress symptoms throughout the 48 h duration of the treatments.

2-DGE analysis of NaCl-responsive proteins in Arabidopsis roots

To investigate the temporal changes of protein profiles during NaCl stress, we carried out 2-DGE analysis of the total proteins in Arabidopsis roots from three biologically independent replicate experiments. A representative gel is shown in Fig. 3-3. Approximately 1,000 protein spots were detected on Coomassie brilliant blue-stained gels and about 600 protein spots were matched between 6 control gels and 6 treatment gels. Quantitative image analysis revealed a total of 215 protein spots that changed their abundance (Vol%) significantly ($P \leq 0.05$) by more than 1.5 fold at one or two time points. We selected 1.5-fold as a threshold value in order to identify most responsive proteins which were consistent with our previous microarray experiments (Jiang and Deyholos, 2006). Essentially arbitrary threshold values ranging from 1.3- to 2.0-fold have been used in previous proteomics studies (Casati *et al.*, 2005; Amme *et al.*, 2006; Parker *et al.*, 2006;Yan *et al.*, 2006). We noted that some protein spots also demonstrated qualitative changes in intensity. For example, spots 21, 24, 28, and 75 were absent in the NaCl-treated gels at one or more time points while spots 26, 62, 82 and 85 were absent in control gels at one or more time points.

LC-MS/MS identification and classification of NaCl-responsive proteins

We arbitrarily selected and excised 89 of the 215 differentially expressed spots described above for tryptic digestion and analysis by LC-MS/MS. From 89 gel plugs excised, we successfully identified 86 proteins (Table 3-1), of which 81 were unique according to the protein identity. Some NaCl-responsive spots were not excised because of their low abundance. We found that at least 23 (26%) of these spots contained peptides that matched proteins from unrelated families (Table 3-1), indicating the presence of multiple proteins in some spots. Conversely, we found that four proteins were identified in more than one spot, although they were excised from the same gel (Table 3-1). For example, L-ascorbate peroxidase (APX1) was identified

from three spots (35, 36 and 61), glutathione S-transferase (AtGSTF2) was identified in two spots (41 and 42), glycosyl hydrolase family 17 protein was identified in two spots (26 and 62) and, glycosyl hydrolase family 1 protein was identified twice (65 and 86). Further examination of electrophoresis patterns indicated that the inferred mass or pI

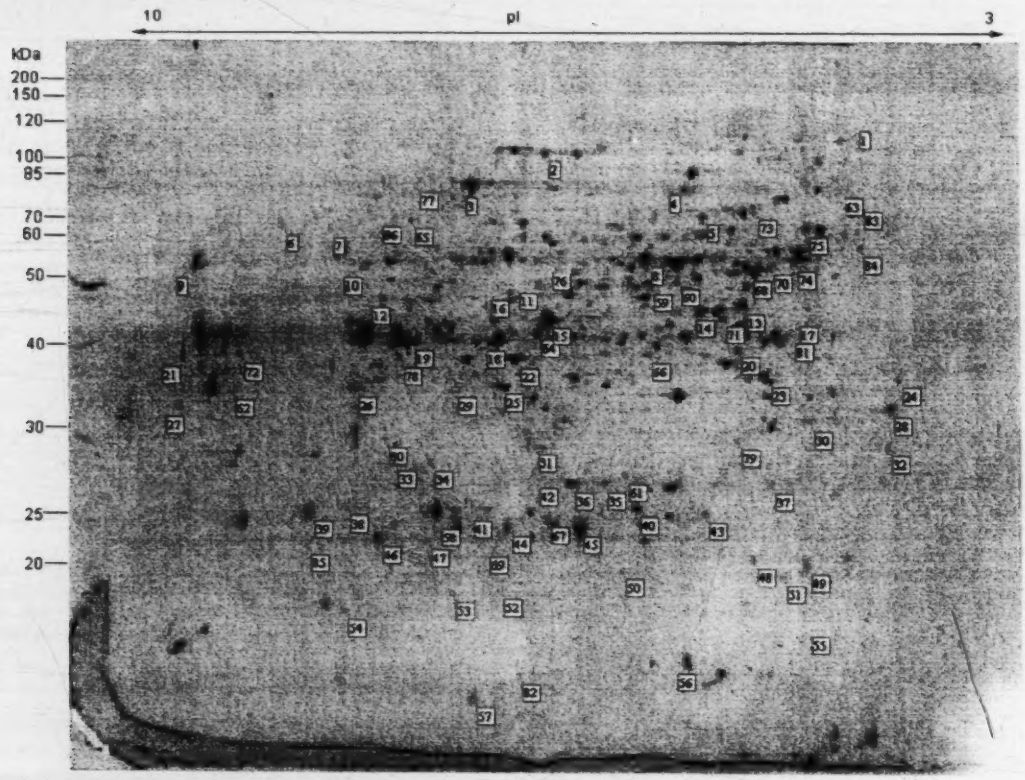

Fig. 3-3. A representative 2-DE gel of Arabidopsis root proteins. 86 of the spots showing at least 1.5-fold change under NaCl treatment at least at one time-point with $p < 0.05$ were analyzed by LC-MS/MS.

values of these spots differed, due perhaps to post-translational modification (PTM) or degradation. PTMs such as glycosylation, phosphorylation, etc, can change the molecular weight and/or charge of proteins. Alternatively, proteins that were present in multiple spots could result from proteins being translated from alternatively spliced mRNAs (Ishikawa *et al.*, 1997). This phenomenon was also reported previously (Holmes-Davis *et al.*, 205; Ndimba *et al.*, 2005). Proteomic studies have also shown that some proteins may be degraded during abiotic stress. For example, rubisco large subunit (RcbL) was detected as 19 different fragments plus the intact protein in NaCl-

treated rice roots (Yan *et al.*, 2006). Similar phenomena have also been reported in pea mitochondrial proteome under chilling stress (Taylor *et al.*, 2005). It is possible that reactive oxygen species (ROS) may also contribute to the degradation of proteins under stress conditions (Desimone *et al.*, 1996; Kingston-Smith and Foyer, 2000).

We classified the identified proteins into 11 categories similar to the convention used by Ndimba *et al.* (2005) (Fig. 3-4). Proteins implicated in energy metabolism (e.g. glycolysis, citrate cycle, electron transport), ROS scavenging and defense, and protein metabolism (e.g. translation, processing and degradation) comprised 52% of the identified proteins. Further examination showed that after 6 h of stress, the abundance of most NaCl-responsive proteins had decreased. In contrast, after 48 h of treatment, we observed that the number of proteins that had increased in abundance was approximately equal to the number of proteins that had decreased in abundance (Fig. 3-4). This suggests that during the initial (6 h) phase of NaCl stress, the synthesis of many proteins was inhibited and/or their degradation increased, while after 48 h, plant roots began to adapt to water deficit and ionic accumulation by synthesizing selected stress-response proteins. This is generally consistent with our previous observations of NaCl-treated roots, in which abundance of transcripts for almost all ribosomal proteins decreased after 6 h, while transcripts for more than 30 peptidases increased at the same time point (Jiang and Deyholos, 2006). However, in our previous microarray analysis, transcripts for ribosomal proteins remained at decreased levels between 6 h and 48 h, suggesting that the increased expression of selected stress proteins we reported here may involve specialized mechanisms of translation that are not reflected in the bulk transcript level of all cellular ribosomal proteins.

Correlation analysis of mRNA and proteins levels

The relationship between gene expression measured at the mRNA level and the corresponding protein level has not been well characterized in plant roots under abiotic stresses. To evaluate the correlation between mRNA and the corresponding protein levels, we compared the differentially expressed protein levels to our previous oligonucleotide microaray data (Jiang and Deyholos, 2006). Tissue for both types of analyses were grown and treated under nearly identical conditions, to minimize the effects of experimental variation in our results. The oligonucleotide probes for two

proteins identified in the proteomic study were not represented in the microarray: ACO2 (At1g62380) and putative tubulin β-2/beta-3 chain (At5g62690). We filtered out those genes whose transcript levels as measured by their signal intensities on arrays did not pass the threshold background intensity and those with low signal in both channels of more than 1/3 of arrays at 6 or 48 h time points. Altogether, 70 and 69 data points were obtained for 6 and 48 h, respectively (Fig. 3-5).

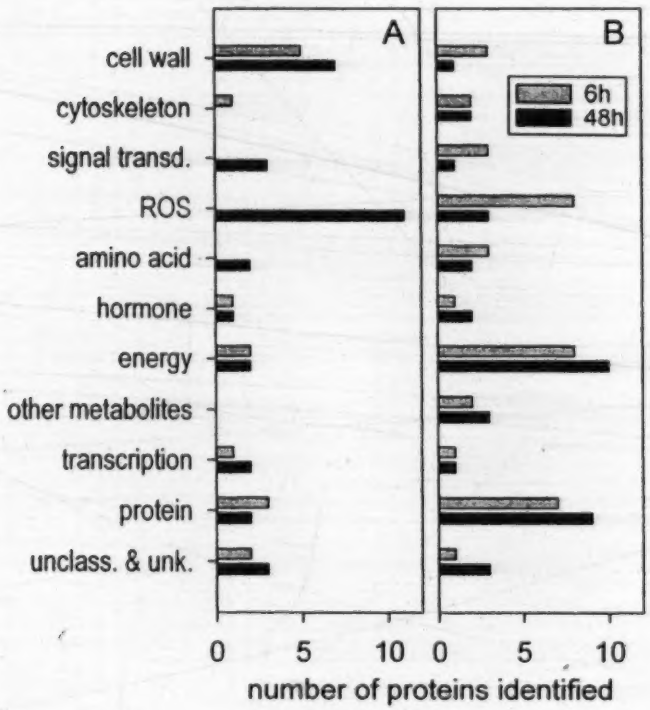

Fig. 3-4. Functional classification of NaCl-responsive proteins. The number of proteins that either (A) increased or (B) decreased by at least 1.5-fold in eleven functional categories is shown for roots exposed to 150 mM NaCl for 6h (grey bars) or 48 h (black bars). Functional categories are named either according to the major metabolic substrate, except for the categories representing processes of signal transduction, transcriptional regulation (transcription), and unclassified or unknown (unclass. & unk.) proteins.

For the proteins whose abundance decreased at 6 h following NaCl treatment,

mRNA of 40 genes (57%, 40 out of 70) also decreased and, this percentage was calculated to be 52% for the 48 h time point. However, for the 22 and 29 proteins that increased in abundance after treated by NaCl for 6 or 48 h, respectively, only 4 (18%) and 6 (21%) genes also increased at the mRNA level (Fig. 3-5). The other spots showed different expression patterns between mRNA and protein at least at one time point (Fig. 3-5). These results support the conclusion of other authors that, in statistical terms, measure-ments of mRNA are not always correlated with protein abundance (Gygi *et al.*, 1999; Tian *et al.*, 2004; Mooney *et al.* 2006; Noir *et al.*, 2006; Yan *et al.*, 2006). Interestingly, a similar pattern was reported in a comparison of 2-DGE and qRT-PCR data for chilling stress in rice: 88% (15 out of 17) of proteins and their corresponding transcripts decreased in parallel following stress, while only 19% (5 of 27) of proteins that increased in abundance following stress had cognate transcripts that also increased in abundance (Yan *et al.*, 2006). Together, these studies suggest that

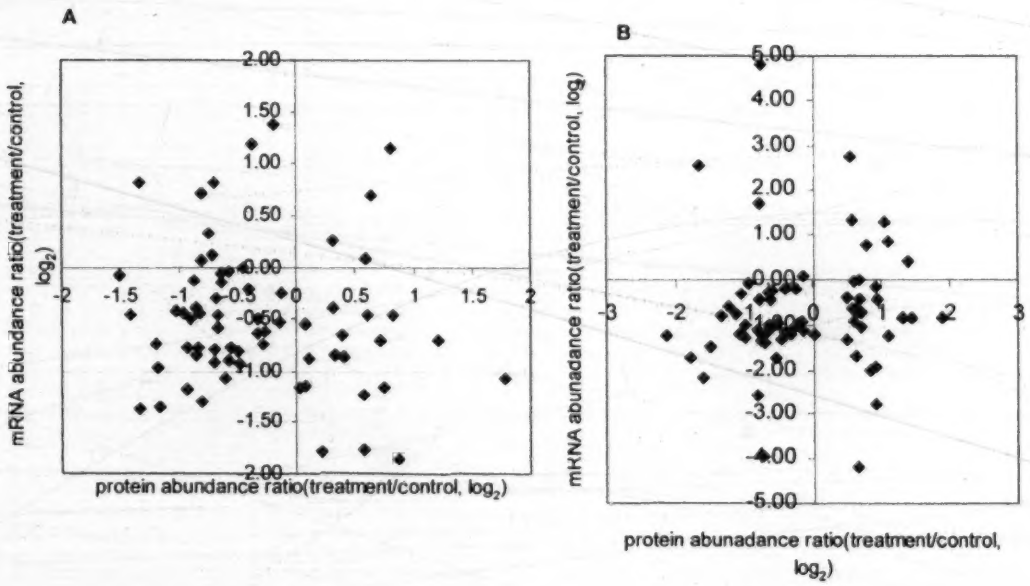

Fig. 3-5. Comparison of NaCl-induced changes in mRNA and cognate protein abundance. The relative change in abundance (treated/untreated) is shown in $\log_2$ scale for proteins extracted from roots treated for (A) 6 h or (B) 48 h, as compared to previously reported (Jiang and Deyholos, 2006) changes in mRNA abundance levels in tissues grown under identical conditions.

transcript abundance may be more directly relevant to expression of genes that are down-regulated following stress than those genes that are up-regulated. However, more study is required to demonstrate the generality of this pattern across a larger sample of proteins, treatments, and measurement techniques.

Discussion

In this study, 81 differentially expressed proteins in Arabidopsis roots in response to salt stress treatment were identified (Table 3-1). These proteins are found to be involved in a variety of plant processes (Fig. 3-4), suggesting an overall adaptation response in roots when challenged by osmotic and ionic stresses induced by high salinity.

Energy metabolism

Under NaCl stress, plants decrease energy metabolism rates to conserve energy and limit further generation of ROS (Moller 2001). We previously reported that the transcript abundance of components of the glycolytic, citrate cycle, mitochondrial respiration, and pentose phosphate pathways (PPP) was generally decreased in NaCl-treated Arabidopsis roots (Jiang and Deyholos, 2006). Therefore, it was not surprising to observe in the present study that the abundance of 11 proteins involved in glycolysis, citrate circle, PPP and electron transport decreased at one or both time points after NaCl treatment (Table 3-1). For example, aconitase (At2g05710), isocitrate dehydrogenase (At1g65930), fumarase (At2g47510) and malate dehydrogenase (At1g04410) are four enzymes of the citrate cycle that each also decreased in abundance following NaCl treatment. In contrast, the abundance of nucleoside diphosphate kinase 1 (NDPK1, At4g09320) , an enzyme converting GTP to ATP, and a mitochondrial malate dehydrogenase (At1g53240) increased at the early phase (6 h) and late phase (48 h) following NaCl treatment, respectively. Differential regulation of structurally related transcripts and proteins has been previously reported in NaCl treated roots, and this may reflect sub-functionalization of related enzymes for optimal activity in different cells or cellular microenvironments (Jiang and Deyholos, 2006). The phosphopyruvate hydratase/enolase (LOS2, At2g36530), which catalyzes the formation of high-energy phosphoenol pyruvate (PEP) from 2-phosphoglycerate in the

glycolytic pathway, decreased after both 6 h and 48 h of stress. Previous reports have shown that mutation in *LOS2* locus results in repression of cold-responsive genes and therefore LOS2 acts as a positive regulator of cold-responsive genes (Lee *et al.*, 2002). The decreased abundance of LOS2 protein that we observed indicates that NaCl-induced perturbation of metabolic flux in glycolysis may be different from that of cold stress. Other energy related proteins, including three proton transporting ATPases, two vacuolar ATP synthases (At1g78900 and At4g11150) and a mitochondrial ATP synthase delta chain (At5g47030) were found to be responsive to NaCl stimulus. Vacuolar H^+-ATPase can generate a proton electrochemical gradient, which is the driving force utilized by the tonoplast Na^+/H^+ antiporter, AtNHX1 to compartment Na^+ into vacuole (Chinnusamy *et al.*, 2005). Vacuolar sequestration of Na^+ is an important and cost-effective strategy for osmotic adjustment that also reduces the Na^+ concentration in the cytosol in plants.

ROS scavenging and detoxifying enzymes

Abiotic stresses induce the production of ROS, which, on one hand can cause damage to cellular components, and on the other hand, can act as signaling molecules for stress responses (Apel and Hirt, 2004). Plants can regulate the ROS level through complex mechanisms such as scavenging them by ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione S-transferase (GST), and superoxide dismutase (SOD), of which 9 proteins were identified to be differentially expressed in this study (Table 3-1). In general, the abundance of these 9 identified proteins was increased upon NaCl treatment. It is proposed that some members of APX, GPX, GST and SOD families are part of the antioxidant system employed by plants (Apel and Hirt, 2004) and, previous microarray results demonstrated that members of them are responsive to various stresses including NaCl, osmotica, drought and cold (Kreps *et al.*, 2002; Seki *et al.*, 2002; Jiang and Deyholos, 2006). The increased abundance of GST, PRX and SOD proteins following NaCl is consistent with the presence of oxidative stress in NaCl-stressed roots. The alleviation of oxidative damage and increased resistance to environmental stresses is correlated with an efficient antioxidative system (Smirnoff 1998). Overexpression of some SOD, APX and GST genes have been shown to improve oxidative stress tolerance in transgenic plants (Allen 1995; Roxas *et al.*, 1997;

Mittler 2002).

GSTs are abundant proteins encoded by a highly divergent, ancient gene family and have protective functions such as detoxification of herbicides, and the reduction of organic hydroperoxides formed during oxidative stress. Recent studies have also implicated GSTs as components of ultraviolet-inducible cell signaling pathways and as potential regulators of apoptosis (Dixon *et al.*, 2002). Our previous microarray analysis indicated that transcripts of at least 19 GST genes increased in abundance in NaCl-treated Arabidopsis roots (Jiang and Deyholos, 2006). Here, we identified proteins of four plant-specific GSTs, AtGST1/ GSTF6 (At1g02930), AtGSTF2/GST4 (At4g02520), AtGST6/GSTF8 (AT2g47730) and AtGST11/GSTF7 (AT1g02920), which all ultimately increased in abundance after 48 h of NaCl treatment, although several of them were slightly decreased in abundance at the initial time point sampled (6 h). Previous studies showed that AtGST1 was up-regulated by a variety of treatments while AtGSTF2 and AtGST6 each showed a selective spectrum of inducibility to different stresses, indicating that regulation of gene expression in this super-family is controlled by multiple mechanisms (Wagner *et al.*, 2002).

APX and GPX can directly detoxify H_2O_2 to H_2O, and previous studies showed that APX1 is a central component of the reactive oxygen gene network of Arabidopsis (Davletova *et al.*, 2005). In Arabidopsis, a family of seven related proteins named AtGPX1- AtGPX7 was identified and several AtGPX genes were up-regulated coordinately in response to abiotic stresses (Milla *et al.*, 2003). AtGPX6 possibly encodes mitochondrial and cytosolic isoforms by alternative initiation and, AtGPX6 transcript showed the strongest responses under most abiotic stresses tested, thus supporting an important role for it in protection against oxidative damage (Milla *et al.*, 2003).

In this study, we observed a NaCl-dependent increase in protein abundance for two class III plant peroxidases, PER22 (At2g38380) and PER23 (At2g38390). Class III peroxidases are plant-specific oxidoreductases that are implicated in various physiological processes such as H_2O_2 detoxification, auxin catabolism, liginfication, suberization, stress response (wounding, pathogen attack, NaCl) and senescence (Hiraga *et al.*, 2001; Passardi *et al.*, 2005). This agrees with our previous results that

Table 3-1. Differentially expressed proteins identified by LC-MS/MS.

spot ID	AGI#	Putative identity	MW (kDa) thr/exp	pI thr/exp	score[a]	PM[b]	C(%)[c]	NCBI Acc#	fold change 6h	48h
energy metabolism										
18*	At3g52930	fructose-bisphosphate aldolase	38.9/37.9	6.05/5.95	711	27	54	NP_190861	0.91±0.12	0.65±0.07
11	At1g65930	isocitrate dehydrogenase (NADP+)/ oxidoreductase	46.1/43.0	6.13/5.93	1433	42	62	NP_176768	0.4±0.10	0.69±0.13
19†	At3g04120	glyceraldehyde-3-phosphate dehydrogenase C subunit(GAPC)	37.0/37.0	6.62/6.62	852	41	52	NP_187062	0.55±0.08	0.46±0.11
43†	At3g55440	triose phosphate isomerase(TPI)	27.4/24.2	5.24/5.39	620	16	49	2009415A	0.62±0.15	0.75±0.09
12*	At2g47510	fumarase(FUM1)/fumarate hydratase	53.5/43.3	8.01/6.79	1004	27	47	NP_182273	0.63±0.20	0.88±0.16
22	At1g04410	malate dehydrogenase/oxidoreductase	35.9/35.9	6.11/5.92	787	55	58	NP_171936	0.55±0.14	0.9±0.21
25†	At1g53240	malate dehydrogenase (NAD), mitochondrial	37.2/33.4	8.54/5.95	726	31	47	AAF69549	1.08±0.12	1.52±0.23
47	At3g27890	NADPH:quinone oxidoreductase(NQR)	21.5/22.4	6.84/6.51	308	10	30	AAD37373	0.45±0.23	1.55±0.17
33†	At5g20080	cytochrome-b5 reductase/ oxidoreductase	36.1/27.6	8.76/6.72	463	16	32	NP_568391	0.92±0.16	0.58±0.14
2*	At2g05710	cytoplasmic aconitate hydratase	98.7/103.2	5.79/5.83	791	28	28	AAD25640	0.96±0.18	0.59±0.11
24	At1g22450	Cytochrome c oxidase subunit,(COX6B)	21.4/33.9	4.31/5.01	83	5	20	AAM63485	0.44±0.16	NDT
8	At2g36530	phosphopyruvate hydratase, enolase(LOS2)	48.0/48.0	5.54/5.54	1038	60	64	NP_181192	0.83±0.28	0.65±0.14
73*	At1g78900	vacuolar ATP synthase(VHA-A) subunit A	69.2/73.6	5.11/5.21	1483	37	52	NP_001031299	1.24±0.19	0.43±0.19
48	At5g47030	ATP synthase delta' chain, mitochondrial	21.5/21.4	6.2/5.2	127	6	16	NP_199514	1.53±0.26	1.39±0.30
31†	At4g11150	V-type proton-ATPase(TUF)	26.3/28.8	6.04/5.86	782	34	56	CAA63086	1.48±0.22	0.62±0.11
76*	At3g03250	UDP-glucose pyrophosphorylase (UGP)	51.9/47.0	5.8/5.83	1128	29	54	NP_186975	0.49±0.19	0.66±0.15
10*	At3g02360	phosphogluconate dehydrogenase	53.9/46.4	7.02/6.91	741	22	35	NP_850502	0.68±0.14	0.57±0.21

57	At4g09320	nucleoside-diphosphate kinase(NDPK1)	16.3/14.2	7.03/6.01	309	14	49	S31444	3.43±0.77	1.01±0.35
80†	At1g47260	mitochondrial gamma carbonic anhydrase	30.2/29.0	6.71/6.79	602	17	46	NP_175159	0.63±0.12	0.7±0.10
ROS scavenging and defense										
67	At1g02930	gluthatione S-transferase(ATGST1)	23.5/23.8	5.8/5.84	344	14	41	CAA72413	0.68±0.14	2.15±0.81
42	At4g02520	glutathione S transferase (ATGSTF2)	24.1/24.5	5.92/5.87	672	25	59	AAC78264	1.02±0.13	1.95±0.18
41*	At4g02520	Glutathione S-Transferase(ATGSTF2)	24.0/24.3	5.93/5.98	734	32	70	1BX9_A	0.79±0.25	1.71±0.20
44*	At2g47730	glutathione S-transferase (GST6)	24.1/23.2	6.09/5.93	582	18	55	AAC63629	0.78±0.10	1.5±0.17
58*	At1g02920	glutathione S-transferase(GST11)	23.6/24.0	6.31/6.19	468	17	48	CAA74639	0.61±0.11	2.06±0.50
36†	At1g07890	L-ascorbate peroxidase(APX1)	27.8/26.4	5.72/5.82	681	50	54	CAA42168	0.62±0.13	1.51±0.07
61†	At1g07890	L-ascorbate peroxidase(APX1)	27.8/26.8	5.72/5.65	387	13	51	NP_172267	1.08±0.25	1.58±0.10
35†	At1g07890	L-ascorbate peroxidase(APX1)	27.8/26.1	5.72/5.71	473	16	54	CAA42168	0.64±0.15	0.64±0.11
21*	At2g38390	peroxidase23(PER23)	38.6/35.4	8.33/9.19	328	18	33	NP_181373	NDT	1.89±0.45
64*	At2g38380	peroxidase22(PER22)	38.7/39.6	5.95/5.87	352	16	20	AAA32842	1.05±0.31	2.15±0.58
52	At4g11600	glutathione peroxidase 6 (ATGPX6)	18.8/20.3	6.59/5.96	239	7	62	BAA24226	0.4±0.17	2.61±1.14
15†	At1g77120	alcohol dehydrogenase(ADH1)	41.9/41.9	5.83/5.80	789	29	43	CAA54911	0.57±0.20	0.6±0.12
69	At3g10920	manganese superoxide dismutase(MSD1)	25.5/22.9	8.47/5.95	470	16	58	NP_187703	1.33±0.35	3.71±1.08
60	At1g51980	metalloendopeptidase	54.6/45.7	5.94/5.47	1126	33	44	NP_175610	0.35±0.18	0.91±0.23
49	At3g56240	copper homeostasis factor	13.1/21.3	4.91/5.17	374	12	53	CAB87423	0.56±0.17	1.41±0.25
13†	At5g03630	monodehydroascorbate reductase, (ATMDAR2)	47.5/43.3	5.24/5.25	1231	43	70	NP_568125	0.88±0.22	0.58±0.13
46	At4g11650	osmotin-like protein (AtOSM34)	27.5/22.2	6.26/6.86	160	6	19	AAM61750	1.16±0.20	1.91±0.28
protein translation, processing and degradation										
23*	At3g09200	ribosomal protein L10	34.2/34.6	5/5.17	411	38	42	NP_187531	0.92±0.15	0.48±0.18
27*	At3g53870	ribosomal protein S3a	27.5/32.6	9.57/9.13	786	31	56	CAB88349	0.83±0.17	0.64±0.07
55	At1g15930	ribosomal protein S12 (RPS12A)	15.7/17.2	5.38/5.11	79	2	16	NP_173045	0.52±0.14	0.29±0.22
7†	At4g34110	poly(A)-binding protein	68.8/67.3	8.19/7.56	901	24	30	CAB80128	0.71±0.10	0.48±0.09
32*	At3g12390	nascent polypeptide associated	22.0/29.0	4.3/5.0	405	17	59	NP_187845	1.65±0.21	0.79±0.11

		complex alpha chain								
75*	At1g77510	protein disulfide isomerase(ATPDIL1-2)	56.6/67.3	4.9/5.16	1204	29	54	NP_177875	NDT	NDT
63†	At1g21750	protein disulfide isomerase(ATPDIL1-1)	55.9/64.3	4.54/5.13	1480	40	53	NP_173594	1.5±0.37	0.33±0.19
5*	At5g09590	mtHSC70-2 (Heat shock protein 70)	73.2/73.6	5.63/5.34	1301	35	40	NP_196521	0.62±0.20	0.32±0.24
40*	At4g31300	20S proteasome beta subunit A (PBA1)	25.2/24.5	5.31/5.58	669	16	49	CAA74028	0.67±0.30	1.5±0.21
29	At5g23540	26S proteasome subunit RPN11	34.4/32.9	6.3/6.19	544	17	41	AAP86672	0.53±0.17	0.4±0.20
17†	At5g43060	cysteine-type endopeptidase	52.4/39.6	5.86/5.11	528	18	24	NP_568620	0.53±0.10	0.85±0.11
38†	At1g56450	endopeptidase	27.7/24.7	6.09/7.01	522	13	52	NP_176040	2.31±0.59	0.58±0.12
78†	At2g46280	eukaryotic translation initiation factor 3 (eIF3I1/TRIP-1)	36.7/35.7	6.5/6.7	800	18	53	NP_182151	0.55±0.09	0.72±0.08
cell wall-related										
6*	At1g47600	glycosyl hydrolase family 1 protein	58.1/58.8	8.34/7.96	976	42	38	NP_175191	1.92±0.40	0.88±0.12
77*	At1g66280	glycosyl hydrolase family 1 protein	60.2/57.5	6.74/6.77	1177	35	37	NP_176802	0.56±0.10	0.51±0.13
86*	At3g09260	glycosyl hydrolase family 1 protein	60.4/62.9	6.95/6.69	925	23	39	AAB38783	0.6±0.09	1.59±0.17
65*	At3g09260	glycosyl hydrolase family 1 protein	60.3/62.9	6.95/6.56	629	23	24	AAB38783	0.54±0.11	3.04±1.01
62	At4g16260	glycosyl hydrolase family 17 protein	37.7/32.4	6.43/8.33	100	4	22	AAL36038	NDC	1.63±0.12
26	At4g16260	glycosyl hydrolases family 17 protein	37.7/32.6	6.43/6.95	793	54	67	AAL36038	NDC	1.59±0.23
56*	At2g21660	glycine-rich RNA binding protein 7(GRP7)	16.9/16.0	5.85/5.44	642	27	76	AAM62447	1.51±0.07	0.85±0.09
45	At4g14630	germin-like protein(GLP9)	23.2/23.2	5.82/5.84	113	8	11	AAD00509	0.92±0.10	1.85±0.21
39	At5g38940	oxalate oxidase (germin protein)-like protein	23.8/24.2	8.62/7.76	146	13	13	BAB08650	1.83±0.19	1.58±0.15
66*	At3g02230	reversibly glycosylated polypeptide (RGP1)	41.1/36.5	5.61/5.54	996	31	66	NP_186872	1.24±0.12	1.87±0.22
hormone-related										
81†	At1g62380	ACC oxidase(ACO2)	36.4/38.3	4.97/5.1	490	12	31	AAC27484	1.31±0.21	0.66±0.09
59†	At1g02500	S-adenosylmethionine synthetase (SAM1)	43.6/45.1	5.5/5.52	740	25	41	AAA32868	0.67±0.12	0.51±0.11
85†	At3g25780	allene oxide cyclase 2 (AOC2)	28.5/22.4	9.19/7.6	141	6	27	NP_566777	NDC	NDC

68†	At3g16470	jasmonate inducible protein(JR1)	48.6/45.1	5.12/5.24	898	27	32	BAB01146	0.83±0.13	1.66±0.15
signal transduction										
83	At1g56340	calreticulin 1 (CRT1)	48.7/61.5	4.46/5.04	807	20	40	NP_176030	0.57±0.10	1.53±0.10
84*	At1g09210	calreticulin 2 (CRT2)	48.4/53.7	4.37/5.02	956	38	43	NP_172392	0.61±0.17	1.62±0.19
28	At1g62480	vacuolar calcium-binding protein-related	16.6/32.6	4.05/4.95	57	2	30	NP_564795	0.79±0.20	NDT
34	At5g20010	small Ras-like GTP-binding protein (Ran-1)	25.6/27.2	6.38/6.45	237	7	35	AAA32851	0.43±0.15	2.69±1.10
amino acid metabolism										
16*	At5g07440	Glu dehydrogenase 2 (GDH2)	45.0/41.4	6.07/5.96	915	32	46	NP_196361	0.63±0.14	1.61±0.18
20*	At1g66200	Gln synthetase(GS)	47.5/39.6	5.97/5.11	499	14	36	1804333C	0.53±0.10	1.53±0.12
71*	At5g14200	3-isopropylmalate dehydrogenase (AtIMD1)	44.3/42.1	5.75/5.35	1051	24	47	AAU90074	1.02±0.35	0.57±0.07
3*	At5g17920	cobalamine-independent Met synthase	84.3/84.3	6.02/6.03	1319	41	35	1U1H_A	0.72±0.10	0.36±0.24
cytoskeleton										
14	At1g49240	actin 8	42.1/42.7	5.37/5.4	670	17	36	AAC49523	0.38±0.26	0.75±0.16
70*	At4g14960	tubulin alpha-6 chain	50.2/47.6	4.93/5.13	682	17	35	CAB10275	1.74±0.21	1.44±0.19
74*	At5g62690	putative tubulin beta-2/beta-3 chain	51.4/48.0	4.7/5.05	1064	39	52	BAC42096	0.54±0.11	0.63±0.04
transcription										
50	At1g73230	(NAC) domain-containing protein	18.0/20.8	5.91/5.66	193	3	30	AAM61406	1.68±0.23	1.89±0.31
53	At1g17880	(NAC) domain-containing protein	17.9/18.9	6.62/6.25	402	12	63	NP_173230	1.05±0.19	1.76±0.20
other metabolism										
79	At1g53580	hydroxyacylglutathione hydrolase, putative	27.0/28.8	5.45/5.26	348	11	34	2GCU_D	0.65±0.10	0.53±0.13
4	At3g60750	transketolase-like protein	81.9/84.3	5.8/5.46	698	20	35	CAB82679	0.83±0.12	0.54±0.11
30†	At5g09650	inorganic pyrophosphatase (AtPPA6)	33.7/31.4	5.71/5.17	592	15	33	AAS57950	0.65±0.10	0.61±0.05
unclassified and unknown										
37†	At2g43090	aconitase C-terminal domain-containing protein	27.1/27.6	6.33/5.19	558	15	54	NP_181837	1.31±0.19	2.46±0.71
82	At4g23670	major latex protein-related	17.6/15.4	5.91/5.86	277	17	42	NP_194098	1.55±0.10	NDC
1	At3g15950	unknown protein	85.2/115.0	4.61/5.09	1094	31	32	NP_188216	0.73±0.12	0.23±0.15
9*	At1g03220	unknown protein	46.4/45.4	8.97/8.79	837	42	47	NP_171821	0.57±0.17	0.59±0.10

51*	At3g52300	putative protein	19.6/20.1	5.09/5.07	888	23	67	CAC07921	1.25±0.18	0.61±0.12
54	At5g10860	unknown protein	22.8/18.2	9.1/7.01	396	9	37	NP_196647	0.75±0.16	1.52±0.11
72†	At2g20360	hypothetical protein	44.0/35.9	9.26/8.45	920	25	42	AAT68351	1.77±0.22	0.81±0.14

The Arabidopsis Genomics Initiative (AGI) locus name retrieved from TAIR (www.arabidopsis.org) by running Blast tool is shown for each protein. Where peptides from a single spot matched proteins from more than one AGI locus (score[a] of >100), only the highest scoring match is shown, and the symbol (*) or (†) is appended to the spot ID for multiple matches within the same (*) or different (†) gene families. Fold change is expressed as a ratio of the Vol% between 150 mM NaCl treated/control roots, and each value represents the mean value ± S.D. of three biologically independent measurements. For some spots, fold-change cannot be accurately calculated because of complete absence of the spot in either treated or control samples; this is noted by the abbreviation NDC (not detected in control) or NDT (not detected in treatment). [a] Probability based molecular weight search (Mowse) score; [b] Number of peptides matched; [c] sequence coverage percentage.* multiple protein matches in the same gene family as the best match shown. † multiple protein matches in different gene families from the best match shown.

the majority of class III peroxidases are responsive, at the transcript level, to NaCl treatment in Arabidopsis roots (Jiang and Deyholos, 2006). The diverse functions of class III peroxidases are in part due to two possible catalytic cycles, peroxidative and hydroxylic,involving the consumption or release of H_2O_2 and ROS (Passardi *et al.*, 2005). Although some functions of these peroxidases appear to be paradoxical, the whole process is probably regulated by a fine-tuning that has yet to be elucidated (Passardi *et al.*, 2005).

SODs catalyze the dismutation of superoxide into oxygen and H_2O_2 and constitute the first line of defence against ROS within a cell (Alscher *et al.*, 2002). Surprisingly, in our previous microarray analyses, transcripts for all three detectable SOD genes decreased in response to NaCl treatment (Jiang and Deyholos, 2006). Among these was manganese superoxide dismutase, MSD1 (At3g10920), which protein we here observed to increase in abundance by 3.7 fold at 48 h post NaCl-treatment (Table 3-1). The contrast in protein and transcript abundance in similarly treated tissues for MSD1 highlights the importance of an integrated proteomic and transcriptomic analysis of gene expression.

Ascorbate (AsA) is a major antioxidant and free-radical scavenger in plants. Monodehydroascorbate reductase (MDAR) is crucial for AsA regeneration and essential for maintaining a reduced pool of AsA. Surprisingly, we found that the abundance of one monodehydroascorbate reductase (AtMDAR2, At5g03630) was down-regulated by NaCl stress, suggesting that although plants require reduced AsA to remove free radicals, the fine tuning of the levels of various antioxidants is also an important consideration in stress responses (Lisenbee *et al.*, 2005).

Protein translation, processing and degradation

Regulation of gene expression is achieved at several levels, i.e. transcriptional, post-transcriptional, translational and post-translational. 13 proteins implicated in protein translation, processing and degradation were identified in our present study. A decrease in bulk *de novo* protein synthesis following NaCl treatment has been detected in Arabidopsis (Ndimba *et al.*, 2005), and our previous microarray data also demonstrated down-regulation of the majority of transcripts for almost all cytosolic and plastidic ribosomal proteins (Jiang and Deyholos, 2006). Similarly, microarray profiling of

Arabidopsis seedlings under hypoxia indicated a repression of bulk protein synthesis followed by selective translation of specific transcripts (Branco-Price *et al.*, 2005). Under dehydration condition, more than 90% of the Arabidopsis mRNAs showing a strong decrease in abundance as detected by microarray displayed reduced polysomal association, indicating a decreased translation of those transcripts (Kawaguchi *et al.*, 2004).

Consistent with these observations, we identified 3 ribosomal proteins (At1g15930, At3g09200 and At3g53870) , whose abundance decreased following NaCl treatment, suggesting that short term NaCl stress represses protein synthesis *in vivo* (Table 3-1). The expression level of a eukaryotic translation initiation factor 3 subunit protein (eIF3I1, At2g46280), which is a homolog of mammalian TGF-beta receptor interacting protein (TRIP), was also found to be decreased in this study. Previous studies showed that eIF3I1/TRIP-1 is involved in brassinosteroid regulated plant growth and development, thereby revealing a putative link between a developmental signaling pathway and the control of protein translation (Jiang and Clouse 2001). We detected several proteins that promote the proper folding of proteins and/or prevent the aggregation of nascent or damaged proteins. Two protein disulfide isomerase-like (PDIL) proteins (PDIL1-1, At1g21750; PDIL1-2, At1g77510), putative nascent polypeptide associated complex alpha chain protein (At3g12390) and mitochondrial HSC70-2 (70-kDa heat-shock cognate, At5g09590) all ultimately decreased in protein abundance in NaCl treated tissues as compared to untreated controls after 48 h, although At3g12390 showed transient increases in abundance after 6 h of NaCl treatment. Members of HSC70 proteins are often involved in assisting the folding of *de novo* synthesized polypeptides and the import/translocation of precursor proteins (Wang *et al.*, 2004). It was proposed that HSC70 might be used as a motor for transporting the precursor protein through the membranes by interacting with the signal peptides (Zhang and Glaser, 2002). The decreased abundance of MtHSC70-2 after NaCl treatment suggests a decrease in the transportation of newly synthesized peptides into mitochon-dria, due partly to a decreased *de novo* protein synthesis under saline conditions.

Cell wall-related proteins

Moderate NaCl stress reduces water availability and leads to the inhibition of plant growth by increasing the threshold pressure for wall yielding in expanding cells or inducing hydraulic limitations to water uptake (Neumann *et al.*, 1994; Steudle 2000). In our current study, four glycosyl hydrolase (GH) family proteins, 3 of which belong to GH family 1 (At1g47600, At1g66280, At3g09260) and one GH family 17 (At4g16260) were identified (Table 3-1). GH1 and GH17 protein families include β-glucosidases and β-1,3-glucanases, respectively, which play important roles in many physiological processes in plants including cell wall remodeling (Bray 2004; Xu *et al.*, 2004). Each of the four GH proteins we identified had a distinctive temporal expression pattern: two of the GH1s decreased in abundance after 6 h, while the third GH1, At1g47600 increased at 6 h; however, after 48 h, At3g09260 (spots 65 and 86) showed increased abundance than controls. The identified two GH17 protein spots (spots 26 and 62) were more abundant in treated tissues than controls at both time points. The diversity of expression patterns suggested that several different physiological processes were represented by these results. Further experiments on the substrate specificity, localization of the enzymes with respect to potential substrates, and the activities of the substrates and hydrolysis products are required to determine the roles of these enzymes in NaCl root responses.

Glycine-rich proteins (GRPs) containing more than 60% glycine have been found in the cell walls of many higher plants and form a group of structural protein components of the wall in addition to extensins and proline-rich proteins (Ringli *et al.*, 2001). GRPs play roles in post-transcriptional regulation of gene expression in plants under various stress conditions, and in most cases, they are accumulated in the vascular tissues and that their synthesis is part of the plant's defense mechanism (Mousavi and Hotta, 2005). We observed that salinity caused a transient increase in the abundance of AtGRP7 (At2g21660) (Table 3-1). Previous studies showed that AtGRP7 transcript level was repressed by ABA, high NaCl and mannitol (Cao *et al.*, 2006). More recent studies suggest that AtGRP7 exhibits RNA chaperone activity and can promote the cold adaptation process in *E. coli* (Kim *et al.*, 2007).

We also identified two germin-like proteins (GLPs)- GLP9 (At4g14630) and

oxalate oxidase-like protein (At5g38940), whose abundance increased in Arabidopsis roots subjected to NaCl treatment (Table 3-1). GLPs exhibit sequence and structural similarity to cereal germins and may be associated with the cell wall (Membre *et al.*, 2000). Although GLPs mostly lack oxalate oxidase activity, some GLPs have SOD activity. GLPs are thought to play a significant role both during embryogenesis and in biotic and abiotic stress conditions. For example, GLP expression was detected in barley roots after exposure to NaCl (Hurkman *et al.*, 1994). A reversibly glycosylated polypeptide (RGP1, At3g02230) was also found to be induced by NaCl treatment. RGP1 is possibly involved in plant cell wall synthesis (Dhugga *et al.*, 1997). Cell wall rigidification, the formation of a physical barrier and a process of class III peroxidase-mediated cross-linking of several compounds (Passardi *et al.*, 2004), would protect plant roots from further dehydration under water deficit.

Hormone-related proteins

Ethylene and jasmonic acid (JA) are hormones whose activity has been previously correlated with environmental stress (Chen *et al.*, 2005; Devoto and Turner, 2005). The majority of ethylene and JA-related transcripts detected in our previous microarray experiments were responsive to NaCl treatment (Jiang and Deyholos, 2006). ACC oxidase (ACO) catalyzes the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. ACO are encoded by a small gene family. We found that ACO2 (At1g62380) increased in abundance at the early (6 h) time-point, later decreased in abundance (Table 3-1). S-adenosylmethionine synthetase catalyzes the production of S-adenosyl-L-methionine (SAM) from L-methionine and ATP. SAM serves as a methyl group donor in numerous transmethylation reactions and is the precursor for the biosynthesis of polyamines and ethylene among other metabolites. In plants, SAM synthetases are encoded by small gene families that contain members that are differentially regulated by NaCl stress (Espartero *et al.*, 1994). Here SAM1 (At1g02500) decreased in abundance following NaCl treatment, which is consistent with our previously reported microarray results (Jiang and Deyholos, 2006).

JA is involved in a wide range of stress, defence, and developmental processes (Devoto and Turner, 2005). We identified one enzyme implicated in JA biosynthesis, allene oxide cyclase2 (AOC2, At3g25780), whose abundance increased upon NaCl

challenge, indicating that increased JA biosynthesis may also be associated with NaCl-responses in Arabidopsis roots. One jacalin lectin family protein (JR1, At3g16470), which is similar to myrosinase binding protein was found to be positively regulated by NaCl. JR1 transcript was strongly induced by wounding and JA (Leon *et al.*, 1998), supporting the concept of cross-talk between various abiotic stresses.

Signal transduction network involved in NaCl stress responses

An increase in NaCl concentration in the extracellular space can be perceived by putative sensors in the cell membrane of Arabidopsis and transmitted to cellular machinery to regulate gene expression (Chinnusamy *et al.*, 2005). Some proteins involved in signal transduction were identified in this study (Table 3-1), i.e. two calcium ion binding proteins (CRT1, At1g56340 and CRT2, At1g09210), a vacuolar calcium-binding protein-related (At1g62480) and a small Ras-like GTP-binding protein (Ran-1, At5g20010). In plant cells, Ca^{2+} is a ubiquitous intracellular second messenger involved in numerous signaling pathways. Modulation of intracellular Ca^{2+} levels is partly regulated by calcium-binding proteins, which, after activated, induce specific kinases. Calreticulin (CRT) is a multifunctional protein mainly localized to the endoplasmic reticulum in eukaryotic cells. Plants have three CRT isoform groups (CRT1/CRT2 and CRT3) and Arabidopsis has 18 CRT proteins and members of the different isoform groups respond differently to applied external stimuli (Persson *et al.*, 2003). The identified CRT1 and CRT2 in this study support that they are the major isoforms, possibly due to an enhanced Ca^{2+}-binding efficiency, and play important roles in Ca^{2+} homeostasis under osmotic stress. Ran is an evolutionarily conserved eukaryotic GTPase, which is likely involved in nuclear translocation of proteins and cell cycle progression (Yang 2002). However, little is known about the function of Ran in plant response to stresses. We found that the abundance of Ran-1 increased after 48 h NaCl treatment, suggesting that Ran could also play a specific role under saline condition.

Amino acid metabolism

The amount of Pro and certain other amino acids is reported to increase under NaCl treatment (Fougère *et al.* 1991; Di Martino *et al.* 2003). We observed that the abundance of four amino acid biosynthesis-related enzymes was influenced by NaCl

(Table 3-1). 3-isopropylmalate dehydrogenase (AtIMD1, At5g14200), which is involved in Leu biosynthesis, and cobalamine-independent methionine synthase (ATCIMS/AtMetE, At5g17920) decreased in abundance following NaCl treatment. However, glutamate dehydrogenase 2 (GDH2, At5g07440), and glutamine synthetase (GS, At1g66200) both decreased in abundance at the 6 h time-point, but increased at the 48 h time-point. GS functions as the major assimilatory enzyme for ammonia and GDH works as a link between carbon and nitrogen metabolism as it can aminate 2-oxoglutarate into glutamate (biosynthetic reaction) or deaminate glutamate into ammonium and 2-oxoglutarate (catabolic reaction). GS and GDH, together with a number of other enzymes play key roles in maintaining the balance of carbon and nitrogen (Miflin and Habash 2002). Recent study showed that salinity-generated ROS signal induces α-GDH subunit expression, and the anionic iso-GDHs assimilate ammonia, acting as anti-stress enzymes in ammonia detoxification and production of Glu for Pro synthesis (Skopelitis *et al*, 2006).

Cytoskeleton

Actin and tubulin dynamics have important functions in cellular homeostasis. The cytoskeleton is rapidly remodeled by various endogenous and external stimuli such as hormones, low temperature, aluminum, and NaCl (Abdrakhamanova *et al.*, 2003; Dhonukshe *et al.* 2003; Sivaguru *et al.*, 2003). For example, the transverse orientation of cortical microtubule arrays in tobacco BY-2 cells was remodeled to a more random arrangement after treatment with 150 mM NaCl for 15 min (Dhonukshe *et al.* 2003). More recent research suggests that NaCl stress compromises the organization of cortical microtubule arrays, in which SPR1 is involved, and inhibits anisotropic growth (Shoji *et al.*, 2006). We found that one actin protein, ACT8 (At1g49240) and one tubulin β-chain (At5g62690) decreased in abundance following NaCl treatment, while tubulin α-6 chain (TUA6) was induced by NaCl (Table 3-1). These observations are consistent with previously reported microarray results (Seki *et al.*, 2002; Jiang and Deyholos, 2006), and although their mechanistic significance is not fully clear, the stress-responsiveness of these common, cytoskeletal proteins calls into question their sometimes designation as house-keeping genes.

Transcription-related proteins

Transcriptional control of the expression of stress-responsive genes is a crucial part of the plant response to various abiotic and biotic stresses. Nascent-polypeptide-associated complex (NAC) is a heterodimeric complex (α- and β-NAC) that can reversibly bind to eukaryotic ribosomes. Rospert *et al.* (2002) suggested that NAC is a negative regulator of translocation into the endoplasmic reticulum and a positive regulator of translocation into the mitochondria. Previous studies found that α chain of NAC in osteoblasts functions as a transcriptional coactivator (Yotov *et al.*, 1998). Here we identified two NAC domain-containing proteins (At1g17880 and At1g73230), which are similar to human transcription factor BTF3 (RNA polymerase B transcription factor 3) and whose abundance increased following NaCl treatment (Table 3-1). Interestingly, transcripts for these proteins significantly decreased in abundance in our previous microarray study (Jiang and Deyholos, 2006). Furthermore, proteomic evidence showed that a rice α-NAC was down-regulated by NaCl and cold stresses (Yan *et al.*, 2005 & 2006). Based on previous microarray results produced by ourselves and others (Seki *et al.*, 2002; Jiang and Deyholos, 2006), it is likely that hundreds of other transcription factors changed in abundance in the tissues we examined, but that these proteins fell below the sensitivity threshold of analysis, and were therefore not detected in our protein gels.

Conclusion

In this study, we performed a proteomic analysis of Arabidopsis roots subjected to non-lethal NaCl treatment for 6 or 48 h, with physiologically defined responses (Fig. 3-1). Symptoms of stress, such as electrolyte leakage and Na concentration continued to increase until at least 48 h, even though considerable remodeling of the proteome had apparently occurred before this time point (Figs. 3-1& 3-2). We identified 81 different NaCl-responsive proteins by LC-MS/MS (Table 3-1). The identified proteins were implicated in a wide range of physiological processes, *i.e.* energy metabolism; ROS scavenging and detoxification; protein translation, processing and degradation; signal transduction; hormone and amine acid metabolisms; cell-wall modifications; as well as cytoskeleton remodeling, which might work cooperatively to re-establish cellular homeostasis under water deficient and ionic toxicity. Some of the proteins identified

here were also identified at the transcript level in previous microarray profiling of Arabidopsis response to NaCl stress (Seki *et al.*, 2002; Kreps *et al.*, 2002; Jiang and Deyholos, 2006) as well as in 2-DGE analysis of Arabidopsis cell suspension cultures (Ndimba *et al.*, 2005). The proteins identified in this study represent only a small part of Arabidopsis proteome responsive to NaCl treatment and many other NaCl-responsive proteins still need to be identified. Considering the limitations of proteomic study based on 2-D gel, i.e. inability to resolve membrane proteins and detect low-abundant proteins, complementary strategies at the transcript, protein and metabolite levels should be used to gain more insight into the intricate network of plant response to high salinity. To address some of these limitations with 2-DGE, alternative gel-free separation methods have been developed. One of them is the direct identification of proteins from complex protein mixtures based on peptide separation via liquid chromatography columns, followed by tandem mass spectrometry. This method has been applied in rice (Wienkoop and Weckwerth, 2006) and *Medicago truncatula* (Larrainzar *et al.*, 2007). Alternatively, sub-proteomics study (Lee *et al.*, 2004; Nohzadeh Malakshah *et al.*, 2007) and metabolic profiling (Kim *et al.*, 2007) have also provided new insights into stress responses. The identification of novel NaCl-responsive proteins provides not only new insights into NaCl stress responses but also a good starting point for further dissection of their functions using genetic and other approaches.

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Chapter 4: Functional characterization of a NaCl-inducible bHLH92 transcription factor

Introduction

Plant stress responses include changes in transcriptional regulation of gene expression, which depend on the interaction of transcription factors (TFs) with *cis*-regulatory sequences. Plants utilize a large number of TFs to regulate gene expression in response to developmental and environmental cues. Transcription factors belonging to different families have been shown to respond to abiotic stress (Chen *et al.*, 2002; Kreps *et al.*, 2002; Seki *et al.*, 2002; Jiang and Deyholos 2006). To activate or repress transcription, TFs must be localized to the nucleus and interact with the basal transcription apparatus. Therefore, environmental signals that regulate the activity of transcription factors may affect any one or a combination of these processes. Regulation of transcription factor activity is achieved by post-translational modifications such as phosphorylation and/or by *de novo* synthesis of TFs. The current analysis of identified TFs suggests that different stress signalling pathways may overlap or converge at specific points (Xiong *et al.*, 2002b; Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu 2001 & 2002).

The basic/helix-loop-helix (bHLH) proteins are a superfamily of TFs in eukaryotes. This family is defined by the bHLH signature domain, which consists of ~60 amino acids with two functionally distinct regions: a basic region involved in DNA binding and an HLH region functioning as a dimerization domain (Murre *et al.*, 1989). bHLH proteins exhibit considerable sequence divergence, except for the conserved bHLH domain (Atchley *et al.*, 1999). According to their phylogenetic relationships, DNA-binding motifs, and functional properties, animal bHLH proteins were divided into six main groups (named as group A to F; Atchley and Fitch, 1997). The core motif recognized by the bHLH proteins is a consensus hexanucleotide sequence known as the E-box (5′-CANNTG-3′). There are different types of E-boxes, and one of the most common is the palindromic G-box (5′-CACGTG-3′). Certain conserved amino acids within the basic region of the protein provide recognition of the core consensus site, whereas other residues in the domain dictate specificity for a given type of E-box (Robinson *et al.*, 2000).

In *Arabidopsis*, 162 bHLH-encoding genes have been identified from the analysis of genome sequences (Bailey *et al.*, 2003; Heim *et al.*, 2003; Toledo-Ortiz *et al.*, 2003). The members of bHLH family of *Arabidopsis* were divided into 21 subfamilies by Toledo-Ortiz *et al.* (2003). Sequence analysis suggests that most of the plant bHLH proteins belong to Group B (Atchley and Fitch, 1997). In addition, recent reports have demonstrated that some plant bHLH proteins can interact with proteins that lack a bHLH domain. In particular, protein complexes with MYB, bHLH, andWD40 proteins were proposed to regulate guard cell and root hair differentiation (Ramsay and Glover, 2005). Compared to animals, only a small number of plant bHLH proteins have been characterized functionally, and in the model plant *Arabidopsis*, less than 40 bHLH genes have been characterized functionally so far. These studies have demonstrated that *Arabidopsis* bHLH genes (AtbHLH) are involved in controlling a variety of biological processes, including phytochrome-regulated light signaling pathways (Ni *et al.*, 1998; Huq and Quail, 2002), flavonoid biosynthesis (Nesi *et al.*, 2000), trichome development (Payne *et al.*, 2000), brassinosteroid response (Friedrichsen *et al.*, 2002), ABA signaling (Abe *et al.*, 1997, 2003), jasmonate (Anderson *et al.* 2004; Boter *et al.* 2004; Lorenzo *et al.* 2004), cold response (Chinnusamy *et al.*, 2003), iron acquisition (Jokaby *et al.*, 2004), auxin conjugate response (Rampey *et al.*, 2006) and stomatal development (Pillitteri and Torii, 2007). More recently, Arabidopsis At*NIG1* (NaCl-inducible gene) encoding a bHLH-type protein containing an EF-hand motif, was found to play a role in salt stress signaling (Kim and Kim 2006) and *AtAIB* (Arabidopsis ABA-inducible bHLH) was identified to be able to positively regulate ABA response (Li *et al.*, 2007).

In our previous microarray analysis of the *Arabidopsis* root response to salt stress, we identified 29 bHLH TFs that showed an at least 2-fold or higher changes in treated plants compared to controls (Jiang and Deyholos 2006). Among these 29 TFs, 15 increased in transcript abundance following salt treatment, whereas 14 decreased in abundance. Among the 15 up-regulated bHLH TFs, *bHLH92* (At5g43650) showed the highest fold-change (150 to 200-fold) by salt treatment across the three time-points examined. In the present study, we investigated further the function of *bHLH92* as a regulator of salt and osmotic stress responses through analyzing its mutant and

overexpression line phenotypes.

Materials and methods

Plant growth and stress treatment

Arabidopsis thaliana wild-type (WT, Col-0), two ABA mutants, *aba1-5* and *abi4-1* in Col genetic background, three sos mutants *sos1-1, sos2-1* and *sos3-1* in Col(*gl1*) background were obtained from *Arabidopsis* Biological Resource Center (ABRC, Ohio, USA). Seeds were surface sterilized by 2.62% household bleach, sowed on ½ x Murashige-Skoog (MS) medium (Caisson Labs) supplemented with 1% sucrose and 0.3% Phytagel (Sigma-Aldrich), and stratified at 4 ℃ for 2 d. Seedlings were grown in 100 X 15 mm petri dishes for 7 d in a growth chamber and then were transplanted into soil (Sunshine Mix 1, Sungro). The growth conditions were: photoperiod of 16 h light/8 h dark, relative humidity (RH) of 70%, 22 ℃ day and light, and the light intensity was ~125 μE m^{-2} s^{-1}. Rosetta leaves, stems, flower buds, young siliques (~7 DAF (days after flowering)) and mature seeds were harvested from WT plants for qRT-PCR analysis.

Hydroponics of *Arabidopsis* was performed as previously reported (Jiang and Deyholos, 2006). Twenty days after stratification (DAS), seedlings were treated with various stress treatments 1.5 h after the dark/light transition in the morning. Salt stress was performed by replacing the growth medium with fresh ½ X MS containing 150 mM NaCl; cold and heat stresses were applied by moving plants in fresh ½ X MS into a 4 ℃ or 37 ℃ growth cabinets (Danby); drought stress was applied by removing all liquid from the hydroponics tanks and leaving plants to dehydrate in the growth chamber; ABA and MeJA treatments were initiated by transferring plants into fresh ½ X MS containing 50 μM (±)-ABA(Sigma-Aldrich) or 10 μM MeJA (Sigma-Aldrich) and 0.05% ethanol; osmotic and oxidative stresses were applied by adding methyl viologen (Sigma-Aldrich) or mannitol (Sigma-Aldrich) into ½ X MS to a final concentration of 10 μM and 200 mM, respectively; $AlCl_3$ stress was applied by adding $AlCl_3$ (Sigma-Aldrich) to a final concentration of 25 μM in ½ x MS containing 200 μM $CaCl_2$ (Merck). Control plants were moved into fresh ½ X MS medium or ½ x MS supplemented with 0.05% ethanol at the same time. For cold and heat stresses, an

independent control was set up by moving plants into a growth cabinet with temperature set at 22 °C. All treatments lasted for 6 h or 24 h with roots and rosette leaves harvested separately from a pool of about 60 plants per treatment. Control samples were harvested at the same time. Samples were flash frozen in liquid nitrogen and kept at -80 °C before use.

Quantitative RT-PCR

Total RNA was extracted from 100 mg of tissue by RNeasy Plant Mini Kit (Qiagen). Dry seed RNA was extracted according to Suzuki *et al.*(2004). Formaldehyde agarose gel electrophoresis was run to examine the RNA quality (data not shown). Reverse transcription (RT) was performed with 2.5 μg of total RNA pretreated with DNase I by DNA-free RNA kit (Ambion) to obtain cDNA with SuperScript III (Invitrogen) or RevertAid H$^-$ MMLV (Fermentas) and oligo(dT)$_{12\text{-}18}$ (Invitrogen) or oligo(dT)$_{18}$ (Fermentas) as the primer in a 20 μL reaction volume. Each cDNA sample was diluted 1:10 in sterile ddH_2O, and 2.5 μL of this dilution was used as template for qPCR. Primers for the PCR reactions were designed by PrimerSelect 5.00 of DNAStar (DNASTAR Inc) or PrimerExpress3.0 (Applied Biosystems) to have a T_m of 60 °C with the length of the amplicons between 80 and 180 bp. We first ran standard curves to check the amplification specificity and efficiency of each primer pair. cDNAs transcribed from 5 μg of total RNA was diluted with ddH_2O to be ¼, 1/16, 1/64, 1/256, 1/1024 and 1/4096. Standard curves for each gene was performed using the dilution serials to cover the range of all template concentrations. Plots of Ct vs. log(input) were used to calculate the PCR efficiencies (E) according to the equation: $E = 10^{-1/slope}$. qRT-PCR was performed with a home-made 2 x SYBR GreenI master mix in 10 μL reactions using the ABI 7500 fast real-time PCR system (Applied Biosystems) according to the manufacturer's instruction. Each PCR reaction contains 2.5 μL of diluted cDNA, 0.4 μM of each of the primers and 5 μL of SYBR Green I mix (20 mM Tris (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.8%, glycerol 0.01% Tween 20, 2% dimethyl sulfoxide (DMSO), 0.2 mM dNTPs, 1×ROX, 0.25× SYBR Green I, 0.03 units/μL Platinum *Taq* DNA polymerase). The initial denaturing time was 2 min at 95°C, followed by 35 cycles consisting of 94°C for 15 s and 60°C for 1 min with a single fluorescence measurement. A dissociation curve was run after the PCR cycles to

examine that only single peak showed for each primer pair. The primers of genes tested for expression levels by quantitative PCR were as follows: *RD29A* (At5g52310), Fwd: 5'-AGGAAGTGAAAGGAGGAGGAGGAATG-3', Rvs: 5'-GTCGCACCACCACC AAACCAG-3'; *bHLH92* (At5g43650), Fwd, 5'-CACGGAACTGAGGATGATGTC-3', Rvs, 5'-GTCGCCGGCTTCTTTCCTTCTCT-3'; *UBQ10* (At4g05320), Fwd, 5'-GGCCTTGTATAATCCCTGATGAA -3', Rvs, 5'- AGAAGTTCGACTTGTCATTA GAAAGAAA-3'. Gene-specific PCR efficiency was used to calculate the expression of target genes relative to the expression of *UBQ10* reference gene (Pfaffl, 2001). qPCR for each gene was done on three biological replicates with duplicates for each biological replicate. The relative transcript level was determined for each sample, normalized using the *UBQ10* cDNA level, and averaged over the three replicates with triplicates for each biological replicate.

Identification of homozygous T-DNA insertion mutants

T-DNA insertion mutants (bulk T_4) developed by the SALK institute (Alonso *et al.*, 2003) and Syngenta (Sessions *et al.*, 2002) were obtained from ABRC (Ohio, USA). Genomic DNA was extracted from 3-week-old rosette leaves of about 14 independent T_4 lines for each T-DNA insertion mutant as well as from WT plants by 2% CTAB method (Chen and Ronald, 1999). PCRs were conducted to screen out putative homozygous mutant lines using two combinations of primers, LB/RP and LP/RP as per the protocol described at http://signal.salk.edu/tdnaprimers.2.html. The primer sequences are as follows: LBb1 for SALK line, 5'GCGTGGACCGCTTGCTGCA ACT3', SALK_033657, LP, 5'-AAATTTTGTTGCCAAGACACG-3'; RP, 5'-GATG GTCTTGAAACTTGTTTCG-3'; SAIL_511_E04, LP, 5'-AAAACCGGATTCAAC CAATTC-3'; RP, 5'- GAAGAAAAATACGGTGGCTCC-3'; LB3 for SAIL line, 5'-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3'.

To confirm the loss of gene expression in putative homozygous T-DNA insertion lines, total RNA was extracted from 4 week old rosette leaves according to Wadsworth *et al.*(1988) with modifications. The extracted RNA was treated with DNaseI (Ambion) before running RT using oligo$(dT)_{12\text{-}18}$ and SuperScriptII (Invitrogen) followed by PCR with gene-specific primers. The primers used are the same as for the overexpression construct (see below). The *ACTIN2* (At3g18780) gene primers used are 5'-GGTCGTA

CA ACCGGTATTGTG-3' and 5'-GATTCCTGGACCTGCCTCA-3'. PCR product was cloned into pBS SK+ based T vector constructed according to Hadjeb and Berkowitz (1996), sequenced using T7 and T3 promoter primers and ABI BigDye reagents (Applied Biosystems) and analyzed in DNAStar (DNASTAR Inc).

Plasmid constructions

General molecular biology experiments were performed according to Sambrook and Russel (2001). Total RNA was extracted from *Arabidopsis* leaves according to Wadsworth *et al.*(1988) with modifications as described previously and treated with DNaseI (Ambion). RT was performed with SuperScriptII (Invitrogen) and oligo$(dT)_{12-18}$ (Invitrogen) using 2 μg RNA as the template. For subcellular localization of TFs, binary vector-pCsGFPBT (GenBank Acc# DQ370426) was restricted with *Nco* I, dephosphorylated with TSAP (thermo-sensitive alkyline phosphatase, Promega) and purified by a PCR purification kit (Qiagen). The coding region of *bHLH92* (744 bp) was PCR-amplified using primers 5'-CATG CCATGGATAACTTTTTTCTAGGT-3' and 5'-CATGCCATGGCTCCACCTCCACCTCCGTAGTCTTTGTAAAAGGAAG-3''. The PCR products were purified, restricted by *Nco* I, purified again and cloned into *Nco* I restricted pCsGFPBT. For screening of positive clones, sequencing and PCR verification of T-DNA insertion into *Arabidopsis* genome, another three primers were designed: pCsGFPBT_TL_Fwd: 5'-CGAATCTCAAGCAATCAAGC-3', pCsGFPBT _sGFP_141Rvs: 5'-GAACTTCAGGGTCAGCTTGC-3', and pCsGFPBT-sGFP_729 Rvs: 5'-CTAGAGGATCCCCTTGTACAG-3'.

For promoter-GUS fusion, a binary vector pCAMBIA1391Z (AF234312) containing a promoterless GUS gene interrupted by a catalase intron and a double cauliflower mosaic virus (CaMV) 35S promoter driving the hygromycin B phosphotransferase gene in the T-DNA region was modified. The double CaMV35S promoter was removed to eliminate its influence on the nearby promoter being analyzed. This was done by excision of the *Bst* XI-*Xho* I fragment (1881 bp) containing the 35S::hyg-35S terminator fragment from pCAMBIA1391Z, blunted by T4 DNA polymerase (Fermentas), and re-ligated to result in the modified vector p0381Z. The promoter region (PbHLH92, 1 kb upstream from the translation initiation codon) of *bHLH92* gene was PCR-amplified using WT genomic DNA as the template and

primers: 5'-CCC AAGCTT GAA ATG TGT GAG GTT TTG TGT -3' (Fwd) and 5'-CATG CCATGG CTTTCTCTCTTAGTGTGTTTG-3' (Rvs). The resulting fragments were digested with *Hin*d III and *Nco* I and inserted into p0381Z.

For overexpression vectors, the coding region of *bHLH92* was cloned by RT-PCR. An *Nco* I site was introduced to the 5' end of forward primers and a *Bst*E II site was introduced to the 5'end of reverse primer. The primer sequences are as follows: Fwd, 5'-CATGCCATGGAT AAC TTT TTT CTA GGT TTG-3', Rvs, 5'-TCGG GGTCA CCTTAGTAGTCTTTGTAAAAGGA-3'. PCR products were purified by a Qiagen PCR purification kit, restricted with *Nco* I and *Bst* EII, and purified again using a Qiagen kit. Then it was ligated into *Nco* I and *Bst*E II digested of pCAMBIA1303 (www.cambia.org, AF234299) in place of *GUS-GFP*. For screening of positive clones, sequencing and PCR verification of T-DNA insertion into Arabidopsis genome, another two primers were designed: pCAM1303_SeqF, 5'CAACCACGTCTTCA AAGCAA3', pCAM1303_SeqR, 5'AAGACCGGCAACAGGATTC3'.

All PCR amplifications were conducted using high-fidelity *Pfx* DNA polymerase (Invitrogen). Five independent positive clones were sequenced using BigDye reagent in ABI3730 sequencer (Applied Biosystems) to confirm that no mismatch or frame-shift mutations occurred.

Arabidopsis transformation and selection

All the above constructs were introduced into agrobacterium GV3101 through the freeze-thaw method and transformed into WT Arabidopsis (Col-0) by floral dip (Clough and Bent, 1998). The PbHLH92-*GUS* construct was co-transformed into *Arabidopsis* with a GUS-free vector pCAMBIA1300 containing only 35S::hyg-Ter within T-DNA region. This was done by culturing agrobacterium harboring the PbHLH92-*GUS* construct and pCAMBIA1303 separately to an OD_{600} of ~0.8 before combined in a ratio of (vol/vol, 2:1) just before transformation. pCsGFPBT and pCAMBIA1303 plasmids were also transformed independently into WT *Arabidopsis* as controls. T_1 seeds from ten independent lines were separately surface sterilized and selected on 10 separate ½ X MS medium plates containing 50 mg/L of hygromycin B (Sigma-Aldrich). After 7 d, putative transgenic seedlings were transplanted into pots containing potting medium and seeds were harvested (T_2), which were further sowed

on ½ X MS medium containing 25 mg/L of hygromycin B, and after 7 d, resistant lines were transplanted into soil pots. Genomic DNA was extracted from 28 d old rosette leaves using 2% CTAB buffer and PCR confirmation of transgene was performed using suitable primers. For co-transformed Arabidopsis, we ran PCR assay of hygromycin-resistant lines using the forward promoter primer (see above) and a *GUS*- specific reverse primer (5'-TGCCCAACCTTTCGGTATAA-3'). For overexpression lines, we first ran PCR confirmation of the integration of T-DNA into T_1 plant genome using the gene-specific forward primer and a pCAMBIA1303 vector-specific reverse primer (5'-AAGACCGGCAACAGGATT C-3'). qRT-PCR screening to detect high expression of transgenes in T_2 generation was performed as described above. Homozygous T_3 seeds were used for phenotypic scoring under various abiotic stresses and GUS staining, while T_2 lines were checked for GFP expression.

GFP observation

Five-day-old seedlings from ten independent T_2 lines were mounted on slides for GFP observation under confocal or epifluorescent microscopes. The roots or hypocotyls were observed and photographed with an Olympus BX51 microscope (Olympus Optical Co.) equipped with a Photometrix CoolSnap fx digital camera (Roper Scientific) and a MicroColor liquid crystal tunable RGB filter (Cambridge Research & Instrumentation, Inc.). For confocal laser scanning microscopy, samples were mounted in water and observed with a Zeiss Axiovert 100M microscope equipped with a Zeiss LSM510 laser module confocal unit (Carl Zeiss). GFP was visualized with the 488-nm line of an Argon laser at 55% of output and 5% transmission, and with either a 505–530-nm bandpass filter.

Histochemical GUS staining

GUS staining was conducted as described by Weigel and Glazebrook (2002) and Salinas and Sanchez-Serrano (2006). 5-d old T_3 seedlings from ten independent lines grown on MS agar plates supplemented with 25 mg/L hygromycin or hydroponically cultured, 20 DAS whole seedlings, flower buds and young siliques were harvested and incubated in a staining solution (0.1M sodium-phosphate buffer, pH 7, 2 mM $K_4Fe(CN)_6$, 2 mM $K_3Fe(CN)_6$, 0.2% Triton X-100 and, 10 mM EDTA, 2 mM X-Gluc (Rose Scientific)) in 50 ml Falcon tubes for 4-10 h at 37°C in the dark and then washed

2 times with 70% (v/v) ethanol at room temperature to remove chlorophyll. Samples were viewed and photographed with a Leica dissecting microscope (Leica Microsystems) equipped with a JVC digital camera. The representative and consistent results were reported. All images were assembled in Adobe Photoshop CS (Adobe Systems).

Stress tolerance tests and REL measurement

Seeds from wild-type (Col-0), homozygous T-DNA insertion lines, or three independent overexpression lines were harvested from plants grown under similar conditions and stress tolerance tests were performed essentially according to Verslues *et al.* (2006). In brief, seeds were surface sterilized and sowed on 1/2 x MS medium (pH5.7) containing 1% sucrose and 0.8% Phytoblend (Caisson Labs) contained in square petri dishes (Fisher Scientific). After stratification at 4 ℃ for 2-3 d, seed plates were placed vertically under the growth condition of 16 h light/8 h dark, RH~70%, light intensity ~130 uE/m^2/s. After 4-5 d, seedlings with similar growth vigor and root length were transferred using blunt forceps onto normal medium plates (1/2 x MS medium, pH5.7, 1% sucrose, 0.8% Phytoblend) or plates supplemented with 100 or 150 mM NaCl, 100, 150 or 200 mM mannitol, or 0.1 or 0.5 μM methyl viologen, or 0.5 μM (±)-ABA and continued to grow vertically for another one week before scored and photographed. Root length was measured either by using a ruler or the free ImageJ software available from http://rsb.info.nih.gov/ij/index.html. The relative electrolyte leakage (REL) assay was performed according to Jiang *et al.* (2007). Each assay was repeated two to three times and data were analyzed using two-tailed Student's *t*-test and significant differences ($p<0.05$) are reported in the figures or figure legends.

Microarray profiling of *bhlh92* mutant

Qiagen Operon 26k *Arabidopsis* oligonucleotide microarrays were used to profile transcriptomes of *bhlh92* T-DNA insertion mutants (*bhlh92-1*) compared to WT control. Total RNA samples from hydroponically cultured roots with three independent biological replicates were extracted using the RNeasy Plant Mini kit (Qiagen). Potential DNA contamination was removed using a DNA-free kit (Ambion), and 5 μg of total RNA was reverse transcribed into cDNAs using Revert-Aid H⁻ MMLV (Fermentas) and primers contained in the Array900 kit (Genisphere). Microarray hybridizations

with dye-flip design were performed using an Array900 kit according to the manufacturer's manual and as described in Jiang and Deyholos (2006). Slides were scanned immediately using ArrayWox (Precision Scientific) and transformed into .tiff images. Data were analyzed using the TIGR TM4 software suite as described in Jiang and Deyholos (2006).

To validate microarray data, qRT-PCR was performed with six selected genes. The primers designed by PrimerExpress3.0 (Applied Biosystems) are as follows. At5g19890 (*PER59*), Fwd, 5'-TAACATCACCGACGTCGTAGCT-3'; Rvs, 5'-TCCCAAGCCGGTAAAGTTGA-3'; At3g02480, Fwd, 5'-GCAAAACGCGAGCTA CCAA-3'; Rvs, 5'- TTGAGCTGAAGCAGCAGCAT-3'; At4g12550 (*AIR1*), Fwd, 5'-GGGACTTGTCCTAAAAATTCCAT-3'; Rvs, 5'-TTCCAAGAATGCTAGCCTTGA CT-3'; At1g20450 (*ERD10*), Fwd, 5'-CGCTGGTTGAAACAGCAACA-3'; Rvs, 5'-GTCTTGGCGTGATAACCTGGAA-3'; At5g56270 (*WRKY2*), Fwd, 5'-ACCGGAGG TCAATACGAGTCA-3', Rvs, 5'-TTCCATGTGTCCCTCGATCA-3'; At5g05340 (*PER52*), Fwd, 5'-TCACCACGGCAGCTTCTTTC-3', Rvs, 5'-GTAGAG CCGCCGTTGAAGAG-3'. qRT-PCR was performed as described previously.

Phylogenetic tree

The protein sequences of 162 members of the *Arabidopsis* bHLH family were retrieved from DATF (http://datf.cbi.pku.edu.cn/) and RARTF (http://rarge.gsc.riken.jp/rartf/). The bHLH DNA binding domain boundary was defined as per Atchley *et al* (1999) and Toledo-Ortiz *et al*.(2003). Only the ~ 60 amino acid residues in the bHLH domain were aligned using ClustalX (v1.83) (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) with a gap opening penalty of 35 and gap extension penalty of 0.75 in pairwise alignment, and a gap opening penalty of 15 and gap extension penalty of 0.30 in multiple alignment parameters parameter settings. Aligned sequences were further examined with gaps introduced manually for optimal alignment. The parsimony tree was drawn using PAUP*4.0 (Sinauer Associates, Inc.). For Fig. 4-1, Adobe Acrobat 7.0 professional was employed to output the alignment file and to display the phylogenetic tree in a PDF format.

Results

Sequence and phylogenetic analysis of *bHLH92* gene

The predicted bHLH92 protein encoded 247 amino acids with a molecular weight of 29.0 kD and an isolelectric point of 10.22 (Fig. 4-1). We further performed multiple alignment of bHLH92 protein to four other bHLH TFs: MYC2/JIN1/bHLH6, MYC3/bHLH5, MYC4/bHLH4 and ICE1/bHLH116, which had been shown to be involved in ABA (Abe *et al.* 2003), jasmonate (Anderson *et al.* 2004; Boter *et al.* 2004; Lorenzo *et al.* 2004) and cold signaling (Chinnusamy *et al.*, 2003). Using the simple modular architecture research tool (SMART, Letunic *et al.*, 2006, http://smart.embl-heidelberg.de/index2.cgi), we identified a bHLH domain for each of the five bHLH TFs we aligned (Fig. 4-1). The amino acid residues within the bHLH domain of bHLH92 are quite divergent from the those of MYC2, MYC3 and MYC4 (Fig.4-1).

Although the signature bHLH domain of the AtbHLH proteins is well conserved, the regions outside of this domain in the remainder of the protein generally are poorly conserved (data not shown). The length of AtbHLH proteins range from 129 aa 1513 aa. Therefore, we used only the ~60 aa encoding the bHLH domain for phylogenetic analysis, as has been the case for previous studies of this kind (Atchley and Fitch, 1997; Atchley *et al.*, 1999; Morgenstern and Atchley, 1999; Ledent and Vervoort, 2001). Using the online SMART tool, we analyzed 162 putative bHLH protein sequences and found that 159 proteins had a typical bHLH domain whereas three, bHLH157, bHLH158, and bHLH159, did not contain a bHLH domain. InterPro (http://www.ebi.ac.uk/interpro/) was used to define the bHLH domains of the latter three bHLH proteins. As shown in the phylogenetic tress, bHLH92 is closely related to bHLH95 (At1g49770) and these two proteins form a relatively independent subclade (Fig 5-2). We also found that neither of these two bHLH gene probes is included in the Affymetrix ATH1 probe set.

In our previous microarray profiling, we found that the *bHLH95* mRNA level was not changed by salt treatment in any of the three time-points examined, whereas *bHLH92* transcript level was greatly up-regulated by salt treatment in *Arabidopsis* roots. Using qRT-PCR, we found that *bHLH92* mRNA abundance in WT control plant roots was very low. To analyze the expression patterns of *bHLH92* in different tissues,

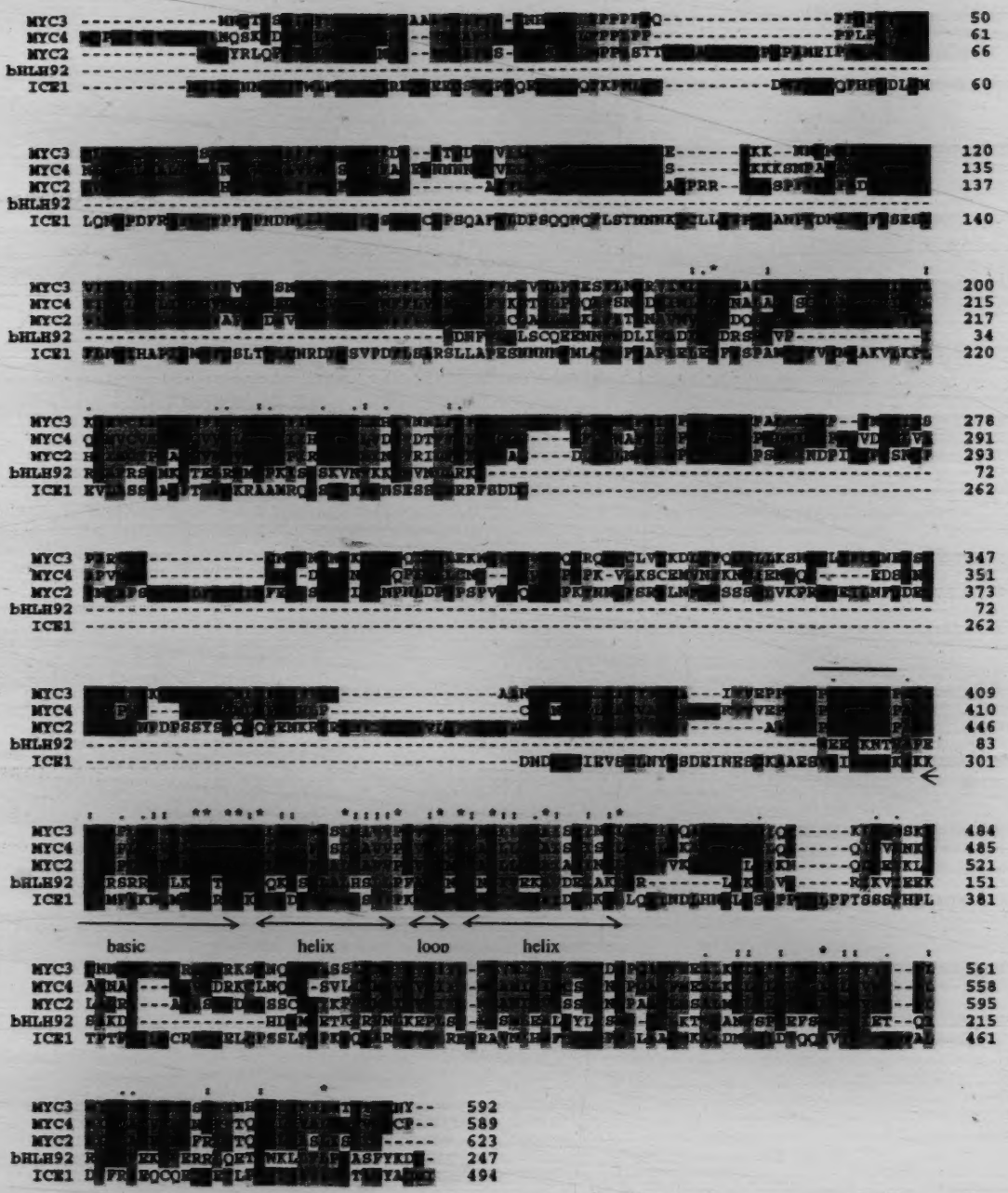


Fig. 4-1. Multiple alignment of bHLH92 and four other *Arabidopsis* bHLH TFs. The symbol "-" in the sequence is the gap introduced for optimal alignment. The asterisks above the sequence indicate identical amino acids, while symbols ':' and '.' indicate conserved and less conserved amino acids, respectively. The bHLH domains at the C-termini are shown by four double-headed arrows under the sequence. The putative nuclear localization signal (NLS) is shown by a line above the sequence.

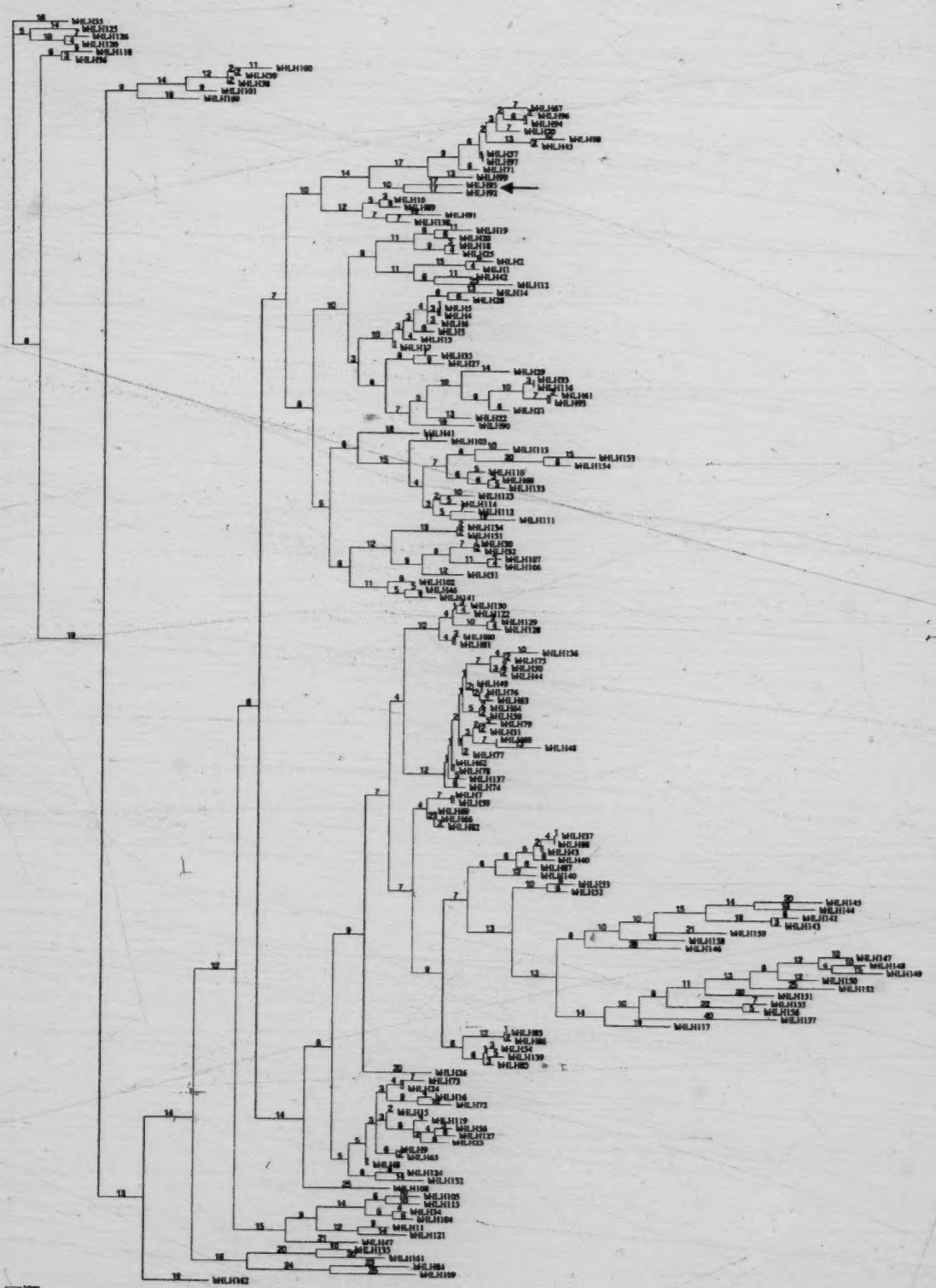


Fig. 4-2. Phylogenetic tree of 162 AtHLHs. The phylogenetic tree was based on the amino acid sequences from bHLH domains only. The numbers on the branches represent branch length. The arrow indicates bHLH92 and bHLH95.

qRT- PCR was used to measure RNA abundance in untreated roots, stems, leaves, flowers, siliques, and seeds. *bHLH92* transcripts were detected at low abundance in all tissues, except flowers and immature siliques, where they were considerably more abundant than in roots. (Fig. 4-3).

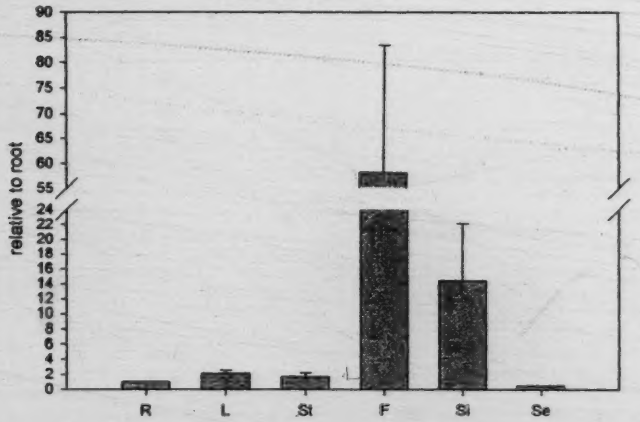


Fig. 4-3. qRT-PCR analysis of *bHLH92* transcript level in various tissues and organs. R, roots; L, rosette leaves; St, stems; F, flower buds; Si, young siliques; Se, dry seeds. The abundance of *bHLH92* in roots was normalized to be 1. Data were from 3 biological replicates with S.D. shown.

Responses of *bHLH92* gene to various abiotic stresses

To investigate the ability of *bHLH92* transcript abundance to be regulated by various environmental stresses, 20 DAS old hydroponic cultured wild-type Arabidopsis plants were treated various stresses for 6 or 24 h and qRT-PCR was used to compare the transcript levels in treated roots in relation to those in corresponding controls. The results showed that *bHLH92* was induced by salt, drought, mannitol, cold, MeJA and aluminum stresses at both 6 h and 24 h time points (2-fold cut-off), and it was also up-regulated by Paraquat at 6 h (Fig. 4-4A). For comparison, we also observed that a stress marker gene *RD29A* (Yamaguchi-Shinozaki and Shinozaki, 1993) was induced by 150 mM NaCl, cold, drought, mannitol, ABA and $AlCl_3$ treatments (Fig. 4-4B).

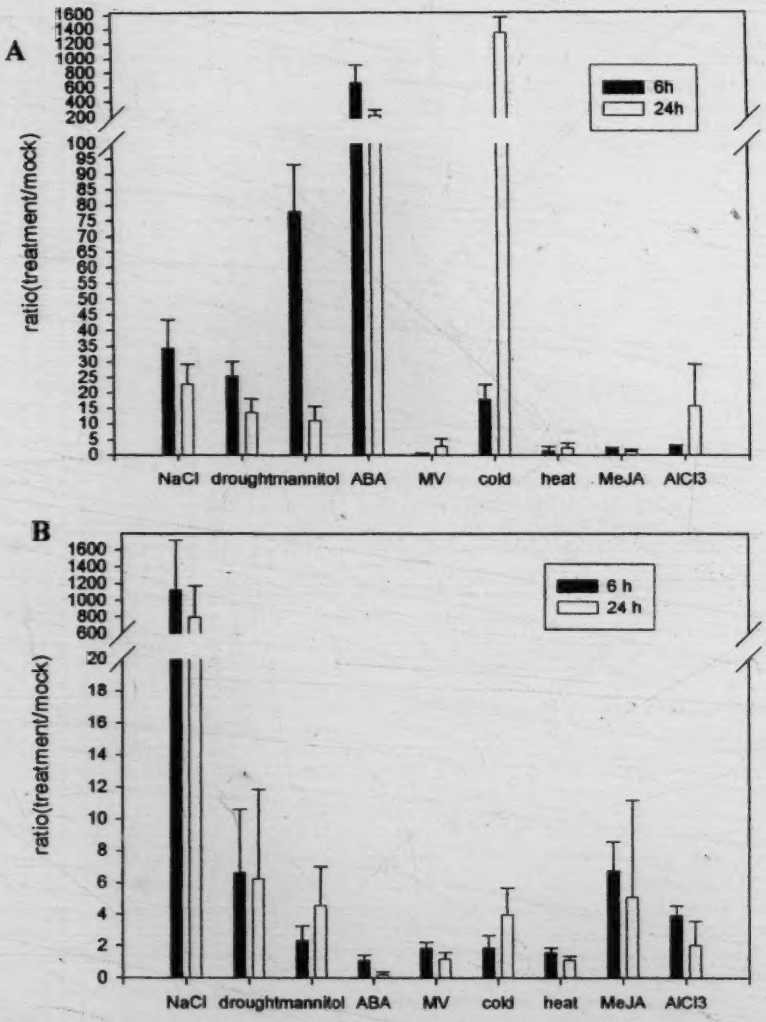


Fig. 4-4. qRT-PCR profiling of *bHLH92*(A) and *RD29A*(B) transcript levels in Arabidopsis roots treated by various abiotic stresses for 6 or 24 h as described in the Materials and Methods section. Values shown are the mean of three independent biological replicates with standard deviation.

Salt-induced accumulation of *bHLH92* transcript in ABA and SOS mutants

ABA regulates several aspects of plant development including seed development, desiccation tolerance of seeds and seed dormancy, and plays a crucial role in the plant's response to abiotic (drought, salinity, cold, and hypoxia) and biotic stresses. Stress-responsive genes have been proposed to be regulated by both ABA-dependent and

ABA-independent signalling pathways (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002; Chinnusamy *et al.*, 2005).

Regulation of cellular ion homeostasis during salinity stress is critical for plant salt tolerance. One of the responses of plant cells to salt stress is the generation of a cytosolic Ca^{2+} transient (Knight *et al.*, 1997) and the subsequent activation of Ca^{2+} sensor protein expression and/or activity. Molecular analysis of *sos* mutants of *Arabidopsis* led to the identification of components (SOS1, SOS2 and SOS3) of a pathway that transduce a salt stress-induced Ca^{2+} signal to reinstate cellular ion homeostasis (Zhu, 2002).

To examine whether the salt-induced expression of *bHLH92* is dependent on the ABA and SOS pathways, we used qRT-PCR analysis to investigate the changes of *bHLH92* in ABA biosynthesis and signaling mutants, *aba1-5, abi4-1* as well as *sos1-1, sos2-1* and *sos3-1* mutants. *aba1-5* is a mutant in which zeaxanthin epoxidase (ZEP), a key enzyme in ABA biosynthesis, is non-functional, while *abi4-1* is a mutation in a

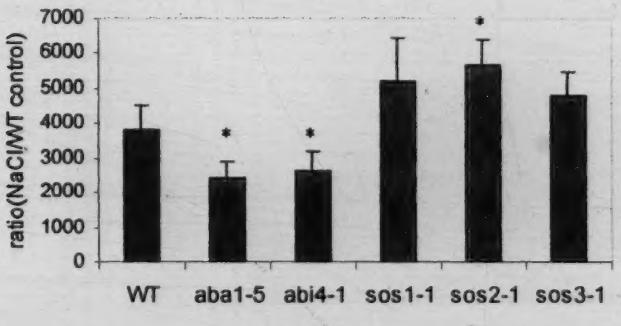


Fig. 4-5. qRT-PCR analysis of *bHLH92* transcript levels in wild-type (WT), *aba1-5, abi4-1, sos1-1, sos2-1* and *sos3-1* mutants treated by 150 mM NaCl for 6 h. Data were the mean values from 3 biological replicates and error bars are the S.D. Asterisks indicate a significant difference ($p < 0.05$) between the mutant and WT.

AP2 domain transcription factor (Finkelstein and Rock, 2002). In the absence of functional *ABA1* and *ABI4*, the fold-induction of *bHLH92* decreased by 37% and 32%, respectively (Fig. 4-5), suggesting that salt-induced *bHLH92* expression is partially dependent on the ABA pathway. We found that the steady-state mRNA level of *bHLH92* in the *sos2-1* mutant was higher than that in WT ($p<0.05$, Fig. 4-5), and in

sos1-1 and *sos3-1* mutants, the difference is insignificant (p=0.05-0.1). Kamei *et al.*(2005) found, through microarray profiling, that *bHLH92* was ~2-fold upregulated by 250 mM NaCl stress treatment for 2 h in *sos3* mutants compared to salt-treated WT plants. The difference between our data and Kamei *et al.* (2005) data may be due to the different concentrations of NaCl used.

Promoter-GUS fusion

To examine the role of a putative promoter region on the expression of *bHLH92* in *Arabidopsis* tissues, we fused the 1 kb region upstream of the translational start codon to the *β-glucuronidase* (*GUS*) reporter gene to determine the developmental, organ-specific and spatial expression patterns it conferred. Promoter analyses can be compromised by interactions between the test promoter and those driving the expression of other genes within the same construct. Information from the pCAMBIA vector series website (www.cambia.org/daisy/cambia/585) has acknowledged problems with some of the promoter-testing vectors. The constitutive CaMV 35S promoter in its various forms contains one or more enhancer sequence that can bidirectionally drive transcription and/or augment the ectopic expression of noncontiguous nearby genes (Xie *et al.*, 2001; Yoo *et al.*, 2005). It has been documented that transcriptional interference can occur between promoters within a construct, depending on their separation distance and relative orientation (Yoo *et al.*, 2005).

To avoid the problem with CaMV35S enhancers, we cloned the 1 kb promoter region (PbHLH92) into a modified binary vector-p0381Z (Fig 4-6A), which does not contain CaMV35S promoter and plant selectable marker gene. We therefore co-transformed our promoter-GUS constructs into WT *Arabidopsis* with another binary vector-pCAMBIA1300, which contains CaMV35S and plant selectable marker gene (hygromycin resistant gene) only within the T-DNA borders (Fig. 4-6A).

Ten independent T_3 transgenic *Arabidopsis* lines were examined to determine the GUS expression patterns conferred by the putative *bHLH92* promoter region. In 5 d old seedlings, a weak GUS expression was observed only in leaf veins (Fig. 4-6B). In 18 d old seedlings, GUS expression was evident in leaf veins (Fig. 4-6C). In inflorescences, GUS expression was visible at the base of young flower buds (Fig. 4-6D). In young siliques (7 d after anthesis, DAA), weak GUS expression was detected only in the seed

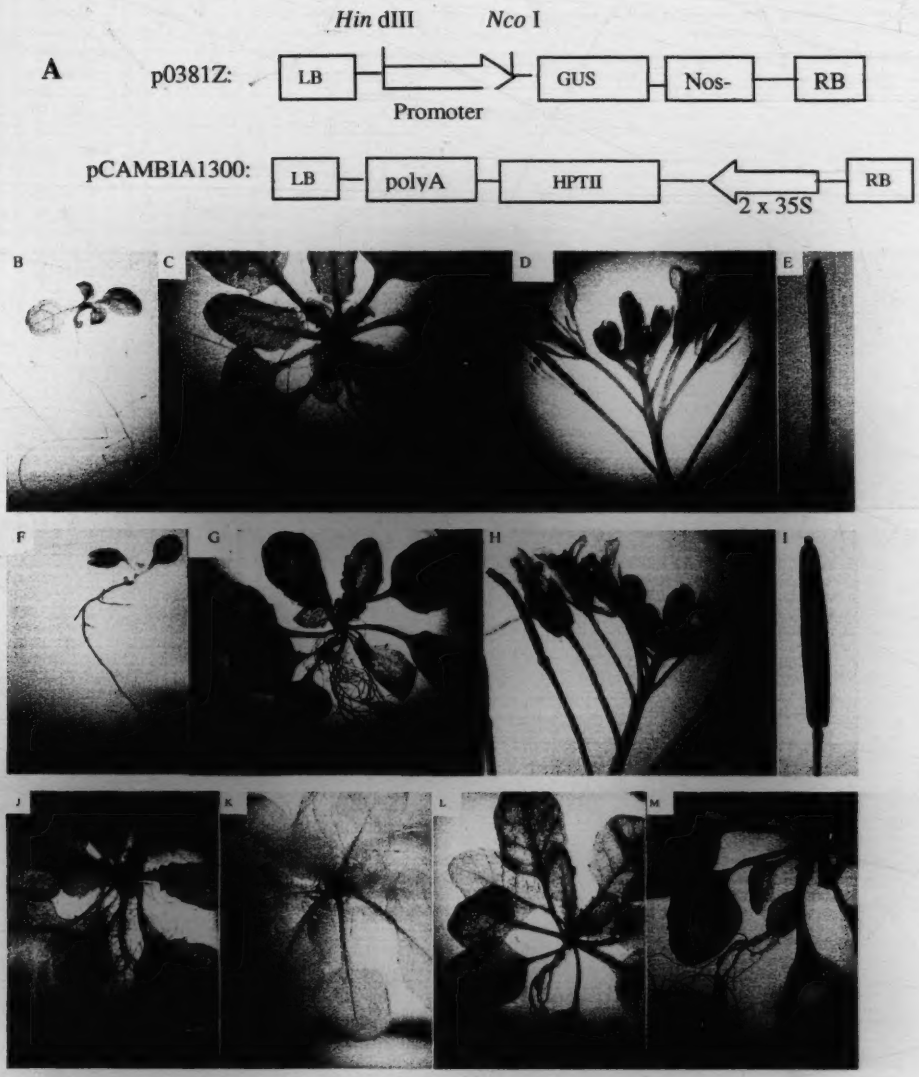


Fig. 4-6. Histochemical localization of GUS activity in representative transgenic *Arabidopsis* plants harboring PbHLH92-GUS construct or pCAMBIA1303 binary vector.

A. Map of T-DNA regions of promoter fusion transformation vector-p0381Z and pCAMBIA1300. LB, left border; RB, right border; Nos-ter, nopaline synthase terminator, CaMV 35S, cauliflower mosaic virus 35S promoter, HPTII, hygromycin phosphotransferase. GUS, β-glucuronidase. B-E, GUS staining patterns in 5-d old seedling (B), 18-d old seedling (C), inflorescence (D), and 7-DAF old siliques (E) harboring PbHLH92-GUS construct. F-I, GUS staining patterns in 5-d old seedling (F), 18-d old seedling (G), inflorescence (H), and 7-DAF old siliques (I) harboring pCAMBIA1303 vector. J-M, GUS staining patterns of 19-d old transgenic line harboring PbHLH92-GUS construct under normal condition (J) or treated by

salt (K), drought (L) and 10 μM JA (M) for 6 h.

pod (Fig. 4-6E). In the control transgenic plants harboring 35S-driven *GUS*, almost all the tissue and organs examined showed strong GUS stainings (Fig. 4-6F-I).

We then examined the GUS expression in 19 d old seedlings treated by salt, drought, cold, heat, ABA or JA for 6 h and compared the staining pattern to the untreated seedling. Our results demonstrated that GUS expression under PbHLH92 was enhanced by salt, drought and JA treatments compared to the mock seedling (Fig. 4-6J-M) while the other abiotic stress treatments tested failed to affect the GUS staining (data not shown).

The putative *cis*-elements contained in the promoter region of *bHLH92* gene were analyzed using PlantCare (Lescot *et al.*, 2002, Table 4-1). We detected three ABRE (ABA-responsive elements, PyACGTGG/T, Yamaguchi-Shinozaki & Shinozaki, 1993) and one CE3 (coupling element). We also found four MeJA-responsive *cis*-

Table 4-1. Putative regulatory sequences identified in the 1 kb upstream region of *bHLH92*.

motif	Sequence	position	strand	Function of regulatory elements
CGTCA-motif	CGTCA	-990	-	MeJA-responsiveness
		-252	+	
		-286	+	
		-182	+	
HSE	AGAAAATTCG	-333	+	heat stress responsiveness
ABRE	TACGTG	-569	+	abscisic acid responsiveness
		-282	-	
	CACGTG	-118	+	
		-92	+	
ARE	TGGTTT	-198	+	essential for the anaerobic induction
		-98	-	
TC-rich repeats	ATTTTCTCCA	-397	+	defense and stress responsiveness
TCA-element	CCATCTTTTT	-767	-	salicylic acid responsiveness
W box	TTGACC	-691	-	wounding and pathogen response
		-141	+	
		-628	-	
CE3	GACGCGTGTC	-985	+	ABA and VP1 responsiveness
WUN-motif	AAATTTCCT	-593	-	wound-responsive element

Putative elements involved in light responses (found in all promoters) are not included in the table. PlantCare: http://bioinformatics.psb.ugent.be/webtools/plantcare/html/

elements, one heat shock element (HSE), one salicylic acid (SA)-responsive element and one wounding responsive element (Table 4-1), indicating that *bHLH92* may respond to these treatments. Interestingly, *bHLH92* promoter region also contains 3 W-box elements (Table 4-1), suggesting its activity might be regulated by some unknown upstream WRKY TF(s).

Sub-cellular localization

To examine the sub-cellular localization of bHLH92, its coding region was fused to the N-terminus of synthetic green fluorescent protein (sGFP) (Chiu *et al.*, 1996) and expressed constitutively under the control of cauliflower mosaic virus (CaMV) *35S* promoter (Fig. 4-7A). The *sGFP* gene alone under the control of the CaMV *35S* promoter served as a control. Stable transformation of WT *Arabidopsis* was performed and 5-7 day old roots from 10 independent T_2 seedlings were checked for GFP expression. The bHLH92-sGFP fusion protein was localized in the nucleus (Fig. 4-7B). With the control vector alone, sGFP signals were distributed in both cytoplasm and nucleus (Fig. 4-7C). This demonstrates that bHLH92 is a nuclear-localized protein, which is consistent with its predicted function as a transcription factor.

Identification of T-DNA insertion lines and stress tolerance tests

In order to test the sensitivity of mutants of *bHLH92* gene to abiotic stress, we obtained and screened out two homozygous T-DNA insertion mutants designated as *bhlh92-1*(SALK_033657) and *bhlh92-2* (SAIL_511E_04). We confirmed by qRT-PCR that the *bHLH92* transcript was undetectable in either of the two mutants, indicating presence of a null mutation (Fig. 4-9A) The T-DNA sites in both mutants are in the first intron (Fig. 4-8B).

Under normal growth conditions, we did not observe significant morphological differences between these null mutants and WT plants grown in soil. However, on normal medium plate, the root elongation of the mutant was significantly longer than that of WT (Fig. 4-8D), suggesting that bHLH92 might negatively regulate root elongation. Root elongation assay of the mutants (compared to wild-type) under NaCl, mannitol, and methyl viologen (Paraquat) stress conditions were performed. Under 100 or 150 mM NaCl treatments, *bhlh92-1* mutant roots were slightly shorter than WT roots, but the difference was not significant (Fig. 4-8C, D). Under 100 or 150 mM

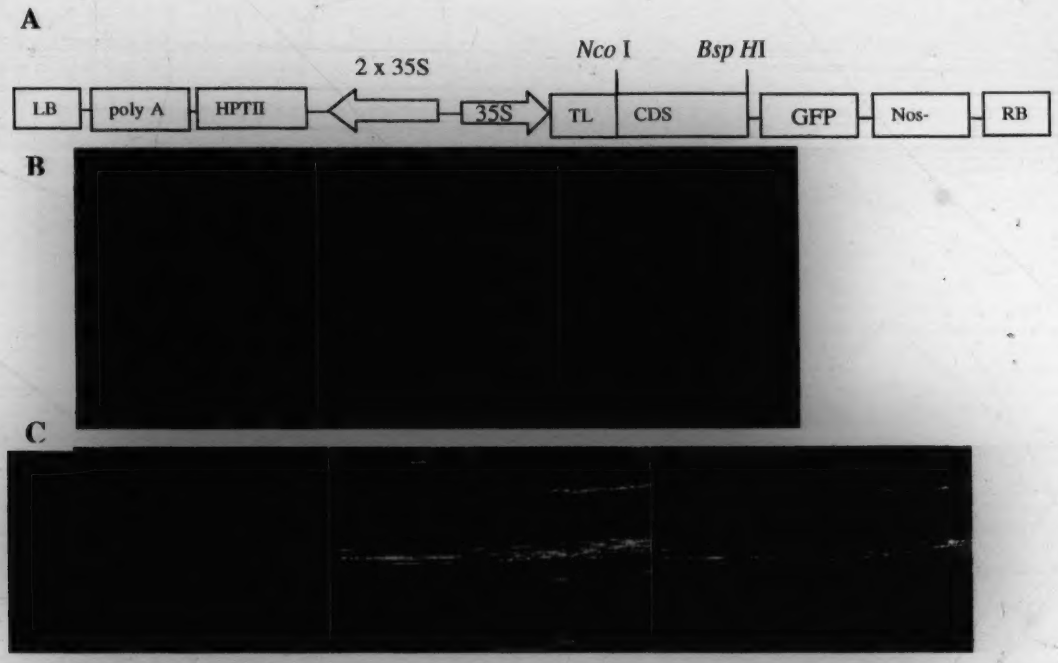


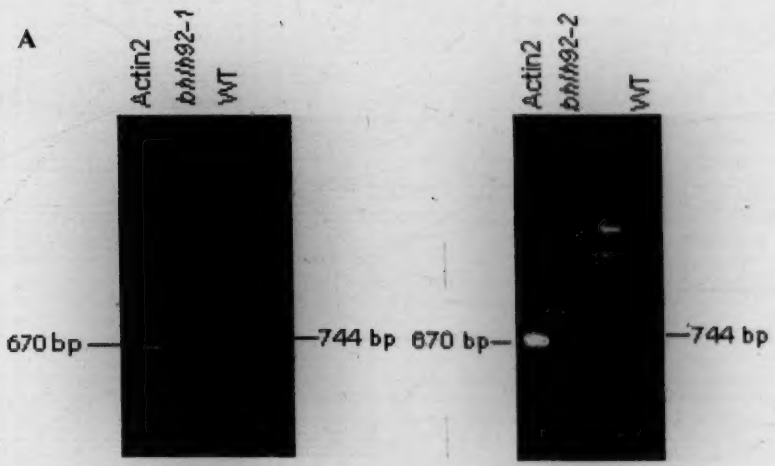
Fig 4-7. Subcellular localization of bHLH92 fused to GFP in *Arabidopsis* root cells.

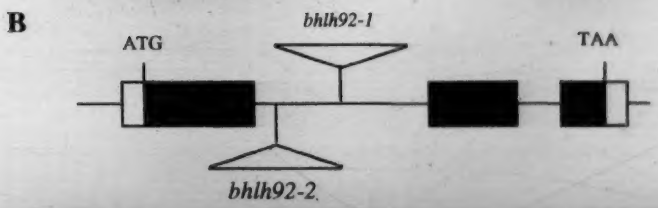
A. Map of T-DNA region of the GFP fusion vector-pCsGFPBT. HPTII, hygromycin phosphotransferase. GFP, green fluorescent protein. TL, translational leader.

B. Subcellular localization of bHLH92-sGFP in root cells of 5-d old transgenic seedlings under confocal microscope.

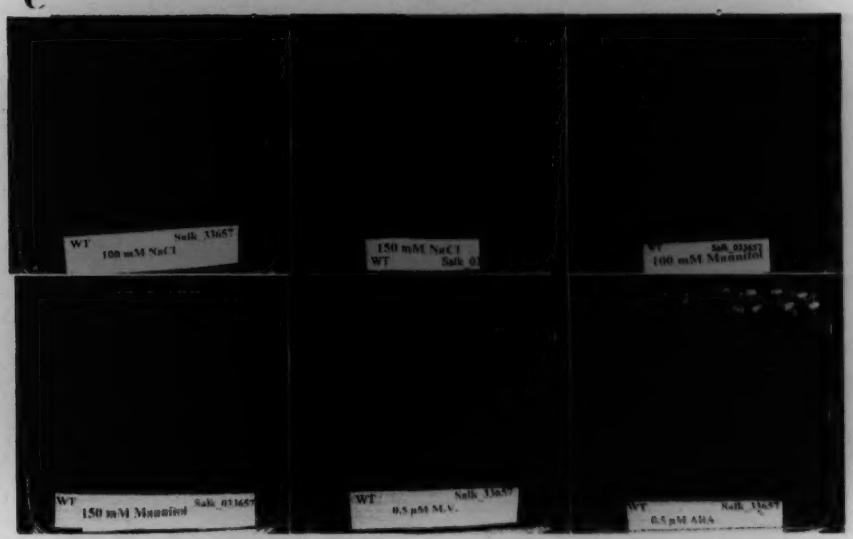
C. Vector control-pCsGFPBT. The fluorescence (left) and bright-field (middle) images are overlaid at right.

mannitol treatment, *bhlh92-1* mutant roots were significantly shorter than WT roots ($p < 0.04$) (Fig. 4-8C, D). No significant difference on root elongation was observed between mutants and WT under oxidative stress or ABA treatment (Fig. 4-8 C, D), although rosette leaves of *bhlh92-1* were a little yellowish on ABA medium (Fig. 4-8C). We further examined the relative electrolyte leakage (REL) of *bhlh92-1* seedlings under 150 mM NaCl treatment for 6 h, and found that REL of the *bhlh92* mutant was significantly higher than that of WT plants ($p<0.05$, Fig. 4-8E). We did not observe any difference between the mutants and WT plants under other types of abiotic stress conditions (data not shown).


A
Actin2
bhlh92-1
WT
Actin2
bhlh92-2
WT
670 bp
744 bp
670 bp
744 bp


B
ATG
bhlh92-1
TAA
bhlh92-2

C


WT
100 mM NaCl
150 mM NaCl
WT
100 mM Mannitol
WT
150 mM Mannitol
WT
WT

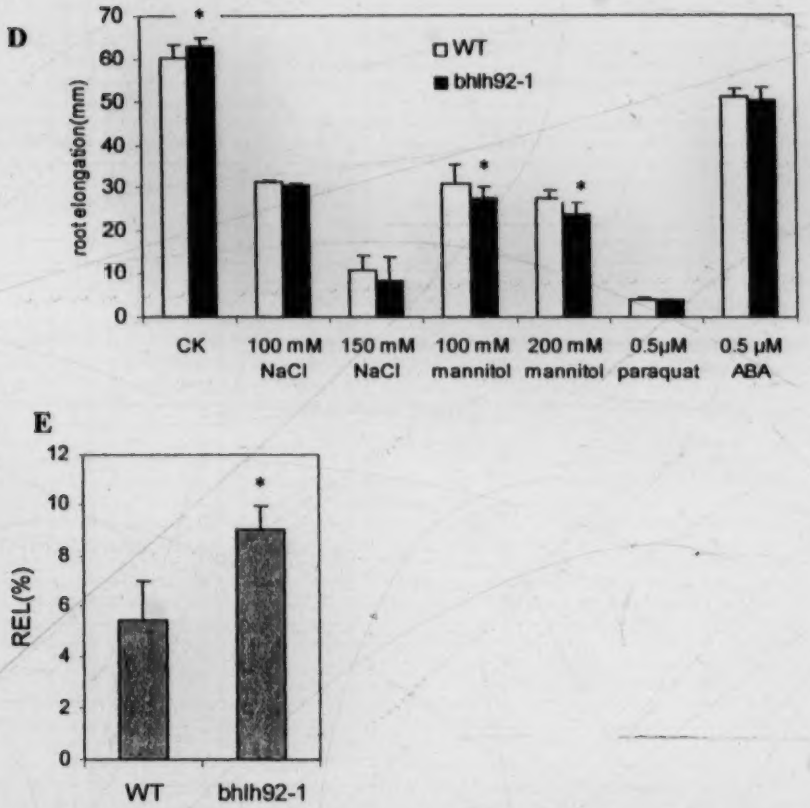
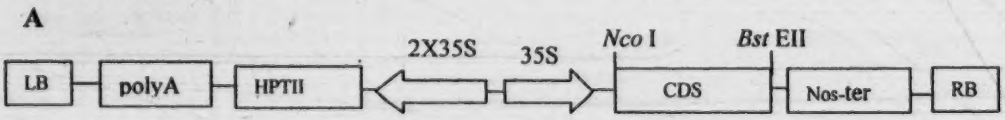


Fig. 4-8. Identification of T-DNA insertion mutants and stress tolerance testing.
A, RT-PCR verification of null mutants of SALK_033657 (*bhlh92-1*) and SAIL_511_E04 (*bhlh92-2*) using *ACTIN2* gene as a positive control. B, Schematic diagrams of *bHLH92* gene and T-DNA insertion sites. Closed boxes represent exons (black, coding regions; white, 5'UTR or 3'UTR), and lines between closed boxes represent introns. The positions of T-DNA insertions are indicated by triangles. C, Stress tolerance test of *bhlh92-1* mutant on ½ x MS medium plates containing 100 or 150 mM NaCl or mannitol, 0.5 μM MV or ABA. D, Quantitative assay of root elongation of *bhlh92-1* under stress treatments. Data are means ± S.D., n=8-10. Asterisks indicate a significant difference ($p < 0.05$) between the mutant and WT. E, Relative electrolyte leakage (REL) from NaCl-treated WT and *bhlh92-1* seedlings following 6 h exposure to 150mM NaCl. Data are means ± S.D., n=6. Asterisks indicate a significant difference ($p < 0.05$) between the mutant and WT.

Over-expression of *bHLH92* gene in *Arabidopsis*

The putative role of bHLH92 in abiotic stress was also investigated by overexpressing the cDNA under the control of CaMV35S promoter in Arabidopsis (Fig. 4-9A). The expression level of the transgene was estimated for 10 independent T_2 lines by quantitative RT-PCR. Lines 2, 3 and 6 were found to have the highest abundance of transcript of the transgene (Fig. 4-9B) and were used for the root elongation assay.

None of the *35S:bHLH92* transgenic plants showed any phenotypic changes compared with control plants under normal conditions. We therefore conducted root elongation assays using homozygous T_3 progeny of these transgenic lines under normal and several types of abiotic stresses, i.e. NaCl, mannitol, Paraquat, ABA and, compared the responses of overexpressing lines to WT control seedlings. On normal medium plate, there is no significant difference between *bHLH92*-overexpressing lines and WT (Fig. 4-9 C, D). Under 100 mM NaCl treatment, *bHLH92*-overexpressing plants were 25% longer then WT roots and, we also detected more lateral roots on *bHLH92*-overexpressing plants than WT plants under salt treatment (Fig. 4-9 C, D). Under 0.1 μM Paraquat treatment, roots of overexpressing lines were also 15.5% longer than WT roots, and *bHLH92*-overexpression lines also had more lateral roots than WT control (Fig. 4-9 C, D). Under 200 mM mannitol treatment, overexpression plant roots were also 28.1% longer than WT control ($p<0.05$, Fig 5-9 C, D). Therefore, overexpression of *bHLH92* increased plants tolerance to NaCl, mannitol, and Paraquat treatments, suggesting that overexpression of *bHLH92* in Arabidopsis can enhance plants' tolerance to high salinity, osmotic and oxidative stresses. We did not observe any significant difference between the *35S:bHLH92* lines and WT under cold or heat treatments (data not shown).



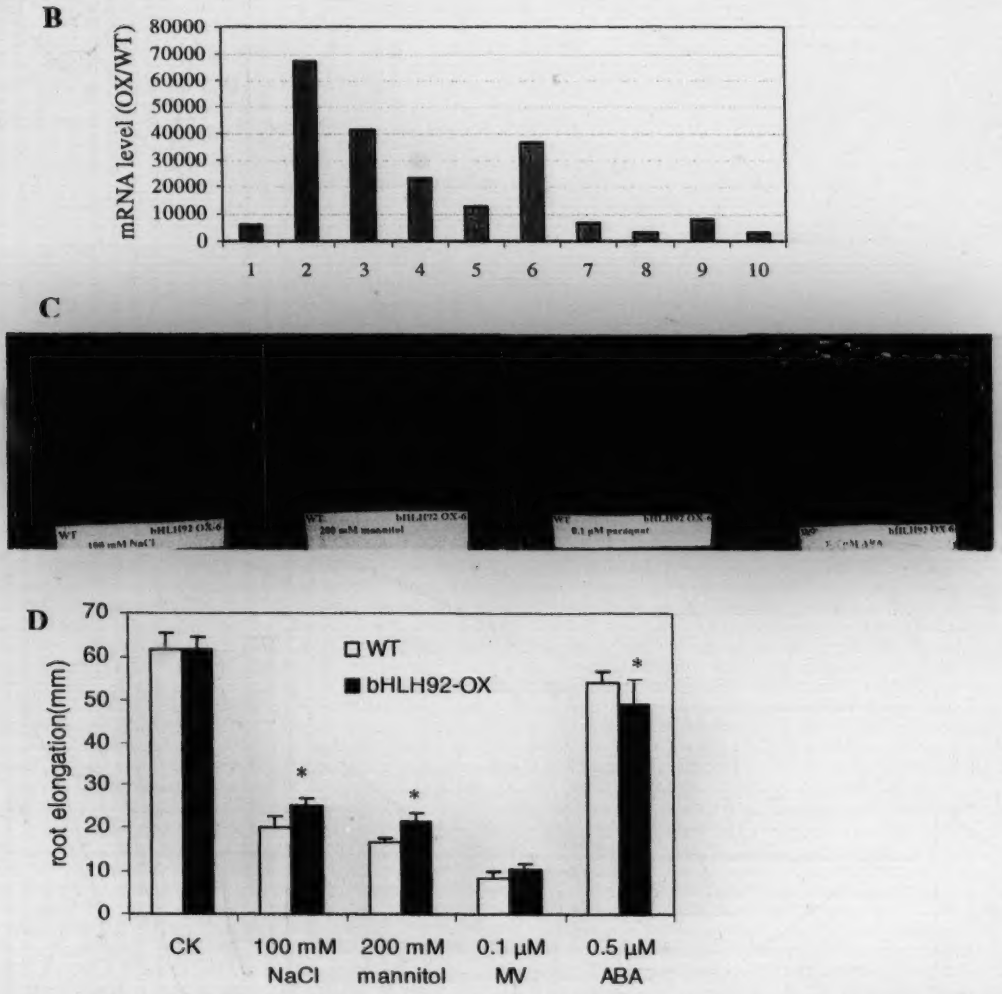


Fig. 4-9. Identification and stress tolerance testing of *bHLH92*-overexpressing Arabidopsis.

A, Map of the T-DNA region of overexpression transformation vector-pCAMBIA1303.

B. qRT-PCR analyses of transcript levels of *bHLH92* in *Arabidopsis* leaves of ten independent T_2 overexpression lines. The ratios were that of genes in overexpression lines compared to that of in wild-type and normalized to the level of endogenous gene UBQ10.

C. Performance of *bHLH92* transgenic line and wild-type plants under salt, mannitol, paraquat or ABA treatments.

D. Effect of 100 mM NaCl, 200 mM mannitol, 0.1μM Paraquat or 0.1 μM (±)-ABA on root elongation of WT and *bHLH92*-overexpressing seedlings. Data are means ± S.D. (n = 8–11). Asterisks indicate a significant difference ($p < 0.05$) between the *bHLH92*-overexpressing plants (OX) and WT exposed to the same treatment.

Microarray identification of bHLH92 target genes

To identify genes that are regulated, directly or indirectly, by the activity of bHLH92, we used a full-genome QiagenOperon 70-mer oligo microarray representing 26, 090 genes (Jiang and Deyholos 2006) to measure transcript abundance in hydroponically cultured *bhlh92* or wild-type plants treated with 150 mM NaCl for 6 h. With a 1.5-fold cutoff ($p < 0.05$), we detected ten and nine genes that decreased- and increased in abundance, respectively, in the *bhlh9-1* mutant compared to WT (Table 4-2). Among the up-regulated gene list, we detected two stress marker genes, *ERD10* and *KIN1*, which encode LEA-like proteins and were proposed to aid in damage repair (Zhu 2001). We also detected two transcription factor genes, one homeobox-leucine zipper family protein gene (*HB53*) and one WRKY gene, *WRKY2*. Interestingly, we detected three class III peroxidase genes (*PER10, PER52* and *PER59*) in the down-regulated gene list (Table 4-2), which suggests that there was an oxidative burst in plants under salinity condition. To validate the microarray data, we used qRT-PCR to measure transcript abundance of five selected genes. The transcript abundance data obtained by microarray analysis corresponded well with those obtained by qRT-PCR (Fig. 4-10).

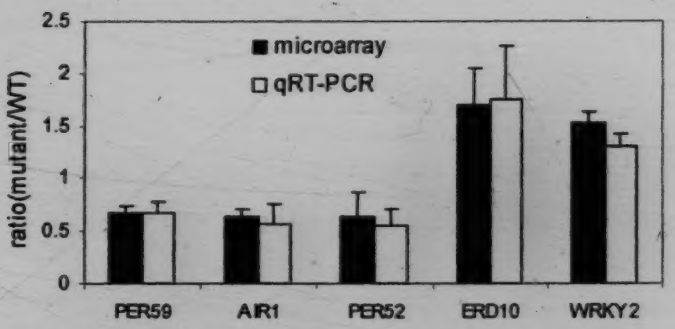


Fig. 4-10. qRT-PCR validation of selected genes from microarray results. Data were from 3 biological replicates with the error bars representing S.D.

We further inspected 1 kb of sequence upstream of the predicted translational start codon of each gene (Table 4-2). We found that *cis*-element G-box (CACGTG) occurs in four of the nine up-regulated gene promoters and only one in ten of the down-regulated genes (Table 4-2). However, for E-box (CANNTG), these numbers increased

to be eight and seven for the up- and down-regulated datasets, respectively (Table 4-2). We propose that the other genes whose 1 kb upstream regions do not contain either G- or E-box may be indirect targets of bHLH92.

Table 4-2. Genes that are regulated by 150 mM NaCl in *bhlh92-1* mutant.

AGI	Putative identity	FC	S.D.	G-box	E-box
Up-regulated					
At1g20450	dehydrin (ERD10/LTI45)	1.70	0.35	+	+
At2g25625	auxin-regulated protein	1.68	0.26	-	+
At1g16850	expressed protein	1.66	0.30	+	+
At4g24411	Unknown protein	1.58	0.36	-	-
At5g66700	homeobox-leucine zipper family protein (HB53)	1.57	0.23	-	+
At4g33550	protease inhibitor/seed storage/lipid transfer protein (LTP) family	1.56	0.25	-	+
At5g15960	stress-induced protein KIN1	1.53	0.18	+	+
At5g56270	WRKY family transcription factor WRKY2	1.53	0.10	+	+
At3g62740	putative beta-glucosidase mRNA, complete cds	1.50	0.37	-	+
Down-regulated					
At4g18250	receptor serine/threonine kinase	0.67	0.22	-	-
At5g19890	peroxidase, putative(PER59)	0.67	0.08	-	+
At3g54120	reticulon family protein (RTNLB12)	0.66	0.22	+	+
At5g24540	glycosyl hydrolase family 1	0.65	0.20	-	+
At4g21920	hypothetical protein	0.65	0.17	-	-
At4g12545	protease inhibitor/seed storage/lipid transfer protein (LTP) family	0.64	0.08	-	+
At4g12550	protease inhibitor/seed storage/lipid transfer protein (LTP) family(AIR1)	0.64	0.08	-	-
At5g05340	peroxidase, putative(PER52)	0.64	0.22	-	+
At5g35940	jacalin lectin family	0.63	0.16	-	+
At1g49570	peroxidase, putative(PER10)	0.49	0.15	-	+

AGI, *Arabidopsis* genome initiative; fold change (FC) is expressed as the mRNA level of *bhlh92* mutant/WT with S.D. representing standard deviation; the presence or absence of G-box or E-box in the 1 kb upstream regions is represented by + or -, respectively.

Discussion

Transcriptional regulation of gene expression is one of the fundamental processes that occurs in response to a variety of signals. Although originally identified to be involved in light response, members of bHLH gene family have been found to play a role in quite distinct biological processes including abiotic stress. For instance, MYC2/JIN1 /bHLH6 has been found to be involved in the dehydration, ABA and JA response

pathway (Abe *et al.* 1997, 2003; Lorenzo et al., 2004). ICE1/bHLH116 regulates the transcription of CBF/DREB1 genes in the cold stress and therefore is an upstream component of the CBF/DREB1 cold-signal transduction pathway (Chinnusamy *et al.*, 2003). We reported here the functional characterization of a salt-induced *bHLH92* gene in the context of abiotic stress response and tolerance.

The transcript of *bHLH92* predominantly accumulated in *Arabidopsis* flower buds and young siliques through qRT-PCR analysis (Fig. 4-3). Compared to the GUS staining pattern (Fig. 4-6B-E), there is difference between mRNA accumulation patterns and GUS expression pattern. This inconsistency between mRNA and reporter activity might be due to the fact that we fused only a 1 kb upstream fragment to the *GUS* reporter gene, and therefore, some *cis*-element(s) may exist beyond the 1 kb upstream region. However, using promoter-GUS fusion, we detected the inducibility of GUS expression by salt, drought and JA (Fig. 4-6J-M), which is consistent with the qRT-PCR profiling result (Fig. 4-4B), suggesting that the 1 kb upstream region contains the necessary *cis*-elements that confer stress response.

ABA is a stress hormone extensively involved in response to abiotic stresses such as salt and drought. At least four systems exist in the regulation of salt- and osmotic stress-inducible genes in *Arabidopsis thaliana* (Shinozaki & Yamaguchi-Shinozaki 1997; Shinozaki *et al.*, 2003). Although most salinity-inducible genes are regulated within an ABA-dependent pathway, some are not dependent on ABA for signalling. A conserved *cis*-acting element, referred to as DRE/CRT, functions in stress-inducible gene expression in an ABA-independent manner (Shinozaki *et al.*, 2003). In ABA-dependent pathways, the ABRE (ABA-responsive element) is a major *cis* -acting element in stress-responsive gene expression (Shinozaki *et al.*, 2003). Genetic analysis of two *Arabidopsis* ABA-deficit mutants, aba1/*los6 and aba3/los5*, in which genes encoding molybdenum cofactor sulfurase (MCSU) and zeaxanthin epoxidase (ZEP) are dysfunctional, showed that ABA is necessary for the salt-stress induced expression of some late-embryogenesis-abundant (LEA) genes (Xiong *et al.*, 2001, 2002a). Our results showed that salt-induced *bHLH92* is partially dependent on the ABA biosynthesis (Fig. 4-5). ABA responsive *cis*-elements (ABREs) are found in the putative *bHLH92* promoter region, but *bHLH92* gene expression is not induced by

ABA treatment according to our criteria (Fig. 4-4B), suggesting a complex mechanism underlying regulating *bHLH92*. It has been shown that a single copy of ABRE is not sufficient for ABA-mediated gene expression, but multiple ABREs or the combination of an ABRE with CE could confer ABA responsiveness to a minimal promoter (Shen *et al.*, 1996; Hobo *et al.*, 1999). We observed that *bHLH92*-overexpressing plants were more sensitive to moderate ABA treatment (Fig. 4-9C), suggesting that ABA is included in the pathway that *bHLH92* works. However, we observed that the null mutant is not more tolerant of ABA (Fig. 4-8C, D). The fact that knock-out mutant and overexpressing did not always give opposite results implies the functional redundancy between bHLH92 and other bHLH TF(s) in some respects or indirect activation of gene expression in the overexpressing plants.

Analysis of *salt overly sensitive* (*sos*) *Arabidopsis* mutants revealed a novel calcium-regulated protein kinase pathway for response to the ionic aspect of salt stress (Zhu 2003). Genetic and physiological data indicate that SOS1, SOS2, and SOS3 function within the same pathway, in which the SOS3–SOS2 complex regulates the expression level of *SOS1* and activates the transport activity of SOS1, which leads to Na^+ tolerance (Qiu *et al.* 2002; Zhu 2003). In addition, salt stress response was also shown to be mediated by SOS-independent signaling pathways with the identification of several protein kinases activated by salt or osmotic stress (Zhu 2001). In *Arabidopsis* seedlings, a 40-kDa kinase similar to ASK1 (serine/threonine kinase 1) was identified as being rapidly activated by hyperosmotic stress in a calcium- and ABA-independent manner. Osmotic stress activation of the *Arabidopsis* 40-kDa protein kinase is not impaired by the *sos3* mutation, implying that its activation is independent of the SOS pathway (Hoyos and Zhang, 2000). Our results showed that salt-induced *bHLH92* transcript accumulation was not significantly different from between *sos2-1 and sos3-1* mutants and WT (Fig. 4-5), but was higher in *sos2-1* mutant than WT, suggesting mutation in *SOS2* affect that salt-responsive *bHLH92* expression. Previous microarray profiling of *sos2-1* and *sos3-1* under 250 mM NaCl treatment revealed that SOS3/SOS2 signalling pathway is independent of the DRE/CRT, ABRE, and MYC/MYB pathways and SOS2 affects the regulation of stress-responsive genes much more strongly than SOS3 (Kamei *et al.*, 2005).

In our experiment, we noted that *bHLH92* was induced by MeJA (Fig 5-4). Not surprisingly, we identified four MeJA-responsive cis-elements in the 1 kb upstream of *bHLH92* gene (Table 4-1). JAs modulate a number of vital physiological processes, including defense against pathogens and insects, wound responses, secondary metabolite biosynthesis, and flower development and fertility (Cheong and Choi, 2003). An *Arabidopsis* bHLH TF, MYC2/JIN1, has also been reported to play major roles in both ABA and JA signaling pathways (Abe *et al.*, 2003; Lorenzo and Solano, 2005; Dombrecht *et al.*, 2007).

Through utilizing T-DNA insertional mutants, we found that *bhlh92* mutant was more sensitive to mannitol treatment compared to WT control, while it did not show any sensitivity to salt stress (Fig. 4-8 C, D). Mannitol can cause osmotic stress in plant cells, making plants lose water to the extracellular environment, whereas high salinity can cause both ionic and osmotic stresses in plant cells (Munns 2005). Microarray profilings have found that many genes are responsive to both mannitol and salt treatment (Kreps *et al.*, 2002), suggesting that some signaling components are shared between them. It is widely believed that there is cross-talk between abiotic stresses (Zhu 2001, 2002; Seki *et al.*, 2003). The observation that *bhlh92* mutant was not significantly more sensitive to NaCl treatment suggests a functional redundancy exists between members of bHLH family. Through phylogenetic tree analysis, we propose that bHLH95 may replace bHLH92 in case bHLH92 is not functional. However, bHLH95 transcript abundance was not responsive to salt treatment.

Overexpression of TFs offers an alternative and complementary strategy to knockout analysis as it is less affected by functional redundancy. In plants, there are several examples in which knockouts of TF genes failed to generate informative phenotypes, but overexpression of these TFs revealed gene functions (Zhang 2003). Overexpression of *bHLH92* using the constitutive CaMV35S promoter slightly increased plant tolerance to salt, mannitol and oxidative stresses (Fig. 4-9), suggesting that bHLH92 is a positive regulator of salt and osmotic stress responses.

Using microarrays, we detected nine up- and ten down-regulated genes in the *bhlh92* mutant (Table 4-2). Among the nine up-regulated genes, *ERD10* and *KIN1* are two well characterized genes involved in abiotic stress response (Kurkela and Franck,

1990; Gilmour *et al.*, 1992; Kiyosue *et al.*, 1994; Weilin *et al.*, 1995). *ERD10* and *KIN1* encode proteins very similar to late embryogenesis abundant (LEA) proteins, and they may play a protecting role under desiccation condition (Wise and Tunnacliffe, 2004), although direct evidence for this is still lacking. As *bhlh92-1* mutant plants were only slightly more sensitive to salt treatment (Fig. 4-8 C, D), the increased abundance of *ERD10* and *KIN1* in *bhlh92-1* mutant may indicate that bHLH92 is a negative regulator of some downstream LEA-like gene expression. The homeobox-leucine zipper family protein gene (*HB53*) is auxin-inducible and may be involved in root development (Son *et al.*, 2005).We identified one WRKY gene, *WRKY2*, which was up-regulated by salt in *bhlh92* (Table 4-2). In WT plant roots, *WRKY2* was not responsive to salt treatment (Jiang and Deyholos, 2006). Further examination showed that *WRKY2* promoter region contain one G-box *cis*-element (data not shown), suggesting *WRKY2* could be a target of bHLH92.

Among the ten genes that were down-regulated genes in the absence of *bHLH92*, three genes (*PER10*, *PER52* and *PER59*) were members of the class III peroxidase gene family were down-regulated (Table 4-2). Class III peroxidases are plant-specific oxidoreductases that are implicated in various physiological processes such as H_2O_2 detoxification, auxin catabolism, liginfication, suberization, stress response (wounding, pathogen attack, NaCl) and senescence (Hiraga *et al.*, 2001). We previously reported that the majority of class III peroxidases are responsive, at the transcript level, to NaCl treatment in *Arabidopsis* roots (Jiang and Deyholos, 2006). However, the specific functions of these PER genes remain to be determined. Furthermore, three protease inhibitor/seed storage/lipid transfer protein (LTP) genes were found to be differentially expressed (Table 4-2). LTPs form a multigenic family whose gene expression in the different plant organs is temporally and spatially regulated (Marion *et al.*, 2007). LTPs can inhibit the growth of fungal pathogens *in vitro* and are also involved in the signalling of defence mechanism of plants against their pathogens (Blein *et al.*, 2002). However, the exact function of LTPs in abiotic stress has not been reported.

More recently, a tobacco (*Nicotiana tabacum*) plastid-resident basic helix-loop-helix protein-NtWIN4 (*N. tabacum* wound-induced clone 4), was found to be converted from a repressor of nuclear transcription into a plastid-resident regulatory factor

through replacement of the DNA-binding domain with a plastid transit sequence during evolution (Kodama and Sano, 2006, 2007). It was also found that bHLH92 protein can be relocated to the chloroplast when the first 30 amino acids are artificially removed (Kodama and Sano, 2006). Therefore, NtWIN4 uncovered a new evolutionary mechanism, although this mechanism is adopted in other cases and species remains unknown (Kleine and Leister, 2007).

In summary, this study shows that bHLH92 plays an important role in salt and osmotic tress responses. This strengthens our understanding of transcriptional regulation during salt and other types of abiotic stress responses in higher plants and provides a base for future work in this area. Future study can be directed to identify upstream gene(s) regulating bHLH92 activity and its interacting partners.

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Chapter 5: Functional characterization of NaCl-inducible *WRKY25* and *WRKY33* transcription factor genes

Introduction

Plants have evolved multiple signaling pathways and defence strategies in response to biotic or abiotic stress. Several classes of transcription factors (TFs) have been implicated in plant response to stress, including AP2/ERF, MYB, WRKY and bZIP (Singh *et al.*, 2002). WRKY TFs contain a WRKY zinc-finger motif, which binds to the W-box motif (C/TTGACC/T) (Eulgem *et al.*, 2000; Ulker and Somssich, 2004). The name of the WRKY family itself is derived from the most prominent feature of these proteins, the WRKY domain, constituted by about 60 amino acid residues. In this WRKY domain, a conserved WRKYGQK heptapeptide is followed by a C2H2- or C2HC-type of zinc finger motif (Eulgem *et al.*, 2000). WRKY transcription factor genes form large families in plants, with 72 members in Arabidopsis. These can be divided into three groups with several subgroups on the basis of their WRKY domains and their phylogenetic clades (Eulgem *et al.*, 2000). Although originally thought to be plant-specific, genes encoding WRKY proteins have been found in two non-photosynthetic eukaryotes: the slime mold *Dictyostelium discoideum* and the protist *Giardia lamblia*. These two lineages evolved prior to the divergence of plants from animals and fungi, indicating an ancient origin of WRKY transcription factors (Zhang and Wang 2005).

Abiotic and biotic stresses are major external factors influencing the expression of WRKY genes (Eulgem *et al.* 2000; Ülker and Somssich 2004). In plants, many WRKY proteins are involved in the defence against attack by phytopathogens such as bacteria, fungi and viruses (Asai *et al.*, 2002; Chen *et al.*, 2002; Dellagi *et al.*, 2000; Kalde *et al.*, 2003, Dong *et al.*, 2003; Yang *et al.*, 1999; Yoda *et al.*, 2002). Furthermore, WRKYs have been found to play essential roles in various physiological processes, including embryogenesis, seed coat and trichome development, senescence, regulation of biosynthetic pathways, and hormonal signaling (Ulker and Somssich, 2004; John *et al.*, 2002; Lagace and Matton, 2004; Xu *et al.*, 2004; Zhang *et al.*, 2004; Zou *et al.*, 2004; Xie *et al.*, 2005). WRKY genes have also been found to be responsive

to abiotic stress. For instance, barley WRKY38 is associated with cold- and drought-response (Mare *et al.*, 2004). Induction of *WRKY* genes has also been observed in *Retama raetam* by drought stress (Pnueli *et al.*, 2002) and in *Nicotiana tabacum* by wounding (Hara *et al.*, 2000). More recently, *Arabidopsis* WRKY75 was reported to be involved in regulating phosphate starvation response and root development (Devaiah *et al.*, 2007). The involvement of WRKY factors in these diverse processes is complicated. Characterization of the expression and the role of individual WRKY members is important and challenging considering the large number of WRKY TFs in plants (Ulker and Somssich, 2004). Recent studies have also revealed that there is cross-talk between abiotic and biotic stress responses and several molecules, including transcription factors (e.g. MYB, MYC) and kinases (e.g. MAPK), as promising candidates for common players that are involved in cross-talk between stress signaling pathways (Anderson *et al.*, 2004; Fujita *et al.*, 2006; Nakagami *et al.*, 2005). In *Arabidopsis*, WRKY29 has been identified as an important downstream component of a MAPK pathway that confers resistance to both bacterial and fungal pathogens (Asai *et al.*, 2002). Recently, Arabidopsis WRKY70 was also identified as a common regulatory component of salicylic acid (SA)- and JA- dependent defense response, mediating cross-talk between antagonistic pathways downstream of nonexpressor of pathogenesis-related genes 1 (NPR1) (Li *et al.*, 2004).

Arabidopsis MAP kinase 4 (MPK4) is an activator of jasmonic acid (JA) and ethylene (ET)-mediated defense, and is a repressor of SA-dependent resistance. In a previously reported study using yeast two-hybrid screening, MPK4 was found to interact with a substrate, MKS1, which in turn interacts with *Arabidopsis* WRKY25 and WRKY33 (Andreasson *et al.*, 2005). In addition, WRKY25 and WRKY33 were phosphorylated by MPK4 *in vitro*, and a *wrky33* knockout mutant expressed elevated levels of *PR1 (PATHOGENESIS-RELATED1)* under a short-day growth conditions (Andreasson *et al.*, 2005). These results suggest that WRKY25 and WRKY33 may function as downstream components of the MPK4-mediated, SA-repressing and JA/ET-activating signaling pathways. Zheng *et al.* (2006) demonstrated that disruption of *WRKY33* results in enhanced susceptibility to necrotrophic fungal pathogens and impaired expression of JA/ET-regulated defense genes. No such phenotypes were

observed in the *wrky25* T-DNA insertion mutants. These results indicate that WRKY33 functions as a positive regulator of JA/ET-mediated pathways and plays an important role in disease resistance to necrotrophic fungal pathogens. More recently, Lippok *et al* (2007) found that rapid, pathogen-induced WRKY33 expression depends on PAD4 (PHYTOALEXIN-DEFICIENT 4), a key regulator upstream of SA, although it does not require salicylic acid (SA) signaling. In addition, WRKY25 functions as a negative regulator of SA-mediated defense responses to the bacterial pathogen *P. syringae* (Zheng *et al.*, 2007).

In our previous microarray study, we identified 18 WRKY TFs, including *WRKY25* (At2g30250) and *WRKY33* (At2g38470) that were induced by 150 mM NaCl treatment in *Arabidopsis* roots (Jiang and Deyholos 2006). This observation emphasizes a potentially important role for WRKY transcription factors in mediating salt stress responses. In this study, we investigated the function of *WRKY25* and *WRKY33* as regulators of salt stress responses. We analyzed their knock-out mutant and overexpression plant phenotypes, which suggest their role in salt, osmotic and ABA signallings.

Materials and methods

Plant growth, stress treatments and mutant isolation

Arabidopsis seeds, either wild-type (Col-0) or mutants, were sown and grown as described in Chapter 4. Leaves, stems, flower buds, young siliques and seeds were harvested from WT *Arabidopsis* (Col-0) plants for qRT-PCR analysis. Hydroponics of *Arabidopsis* was performed as previously reported (Jiang and Deyholos, 2006). At 20 DAS (days after stratification), seedlings were treated with various stress treatments as described in Chapter 4. All treatments lasted for 6 and 24 h with roots and rosette leaves harvested and pooled separately; control samples were harvested at the same time. Samples were flash frozen in liquid nitrogen and kept at -80°C until use.

Arabidopsis mutant seeds, *aba1-5, abi4-1, sos1-1, sos2-1* and *sos3-1* were obtained from *Arabidopsis* Biological Resource Center (ABRC, Ohio, USA) and hydroponically cultured as above for 20 d with roots harvested for qRT-PCR analysis. T-DNA insertion mutarts (bulk T_4) developed at the Salk Institute (Alonso *et al.*, 2003)

and Syngenta (Sessions *et al.*, 2002) were obtained from ABRC. The homozygous T-DNA insertion lines for each gene were identified by PCR with primers designed according to http://signal.salk.edu/tdnaprimers.2.html. The primer sequences are as follows: LBb1 for SALK lines, 5'-GCGTGGACCGCTTGCTGCAACT-3'; 5'-GTCACAACAATC-CGGAAGAAC-3' (LP) and 5'-CAAACTGGCATTGTACA CAGC-3' (RP) for SALK_006603; 5'-GTTGAGATTGTAGCTGCCAGG-3' (LP) and 5'-AGAATGA-TCGACGCAAAACC-3'(RP) for SALK_136966; LB3 for SAIL line, 5'-TAGCAT-CTGAATTTCATAACCAATCTCGATACAC-3'; 5'-TGTTTGGTT GATCA-TCTTTCCTC-3' (LP) and 5'-GGAGGAACAACCTGAGATGAAG-3' (RP) for SAIL_529_B11.

Genomic DNA was extracted from 4-week-old rosette leaves of T_4 lines for each T-DNA insertion mutant as well as from WT plants by 2% CTAB method (Chen and Ronald, 1999). PCR was conducted using two combinations of primers (LBb1/RP and LP/RP) as per the protocol described in http://signal.salk.edu/tdnaprimers.2.html. To confirm the putative homozygous T-DNA insertion lines, total RNA was extracted from ~ 4 week old rosette leaves according to Wadsworth *et al.* (1988) with modifications, and dissolved in 20 µl RNase-free water. The extracted RNA was treated with DNaseI (Ambion), followed by reverse transcription using oligo$(dT)_{12-18}$ and SuperScriptII (Invitrogen), followed by PCR using gene-specific primers (see below). PCR products were cloned into a pBS SK+ (Stratagene)-based T vector and sequenced using T7 and T3 promoter primers (Invitrogen). General molecular biology experiments were performed according to Sambrook and Russel (2001).

Quantitative RT-PCR

Total RNA was extracted from ~100 mg of tissue using the RNeasy Plant Mini Kit (Qiagen). Dry seed RNA was extracted according to Suzuki *et al.*(2004). qRT-PCR was performed as described in Chapter 4. Data were analyzed in Sequencing Detection System (v1.4) and Microsoft Excel 2003.

The primers used for measuring transcript abundance were as follows: *RD29A* (At5g52310), Fwd, 5'-AGGAAGTGAAAGGAGGAGGAGGAATG-3', Rvs, 5'-GTCGCACCACCACCAAACCAG-3'; *WRKY25* (At2g30250), Fwd, 5'-TGGTTCTTC

CGGCGTTGACTGTTA-3', Rvs, 5'-GTGAAATCGGAAGAGGTGG-TGGTTG-3'; *WRKY33* (At2g38470), Fwd, 5'-TTACGCCACAAACAGAGCAC-3', Rvs, 5'-CCAAAAGGCCCGGTATTAGT-3'; *UBQ10* (At4g05320), Fwd, 5'- GGCCT TGTATAATCCCTGATGAA-3', Rvs, 5'- AGAAGTTCGACTTGTCATTA-GAAA GAAA-3'. qRT-PCR for each gene was assayed on three biological replicates with duplicates or triplicates for each biological replicate. The relative transcript abundance was determined for each sample, and was normalized using the UBQ10 cDNA level as described in Chapter 4.

Plasmid construction and plant transformation

Total RNA was extracted from *Arabidopsis* roots according to Wadsworth *et al.*(1988) with modifications. After treated with DNaseI (Ambion), 2 μg of RNA was reverse transcribed using SuperScriptII (Invitrogen) and Oligo(dT)$_{18}$ (Invitrogen). All primers were manually designed and restriction sites were analyzed using DNAMAN (v4.0, Lynnon Biosoft). For subcellular localization of TFs, the coding regions (CDS) of *WRKY25* (1182 bp) and *WRKY33* (1560 bp) were amplified by RT-PCR using the following primers: 5'-CATG CCATGGAT TCTTCCACTTCTTTCACCGA-3' (Fwd) and 5'-CATGCCATGGCAGCTCCACCTCCACCTCCCGAGCGACGTAGCGCGGT TGG-3' (Rvs) for WRKY25, 5'-CATG CCATGGCTGCTTCTTTTCTTACA-3' (Fwd) and 5'-TTAGGTCATGACAGCTCCACCTCCACCTCCGGGCATAAACGA ATCGAA AAA-3'(Rvs) for WRKY33, 5'-CATG CCATGGCGGCGACATCTTCC TCT-3'(Fwd) and 5'-TTAGGTCATGATAGCTCCACCTCCACCTCCAAGCTC GGGCTGCATTTGCTC-3' (Rvs) for Arabidopsis *ACS1*(At2g43750). The CDS regions were amplified from WT cDNAs using *Pfx* DNA polymerase (Invitrogen) and PCR products were purified using a Qiagen PCR purification kit, restricted by *Nco* I and/or *Bsp* HI, purified again and cloned into *Nco* I restricted pCsGFPBT with a Gly-Ala- rich peptide linker between CDSs and sGFP.

For promoter-GUS fusions, we started with binary vector pCAMBIA1391Z and modified it to be p0381Z as described in Chapter 4. The promoter regions of *WRKY25* and *WRKY33* genes were PCR-amplified using WT genomic DNA as the template with the following primer pairs: 5'-CCCAAGCTTCTACTTCAAATACATATATAC-3' (Fwd) and 5'-CATG CCATGG ACG AAA AAT GGA AGT TTG TTT-3' (Rvs) for

WRKY33 promoter, 5'-AGGGATCCACACTTGACGATTTATCAC-3' (Fwd) and 5'-CGAATTCGATGGTCTTTAATAAAGGAG-3' (Rvs) for WRKY25 promoter. The resulting fragments were digested with *Hind* III and *Nco* I and inserted into the p0381Z binary vector.

For overexpression, coding regions of *WRKY25* and *WRKY33* were cloned by RT-PCR using primers 5'-CATG CCATGGTAATGTCTTCCACTTCTTTCAC-3' (Fwd) and 5'-TCGG GGTCACC TCACGAGCGACGTAGCGCGG-3' (Rvs) for *WRKY25,* 5'-CATG CCATGGCTGCTTCTTTTCTTACA-3' (Fwd) and 5'-TCGG GGTCACC TCAGGGCATAAACGAATCGAA-3' (Rvs) for *WRKY33.* PCR amplifications were conducted using high-fidelity *Pfx* DNA polymerase (Invitrogen) and, PCR products were purified by a Qiagen PCR purification kit, restricted with *Nco*I and *Bst* EII, and purified again using a Qiagen kit before ligated into *Nco* I and *Bst*E II digested of pCAMBIA1303 in place of *GUS-GFP*. All the inserts were sequenced using BigDye reagent (Applied BioSystems) and analyzed using DNAStar (DNASTAR Inc) to verify that no mismatches or frame-shifts had occurred.

All constructs plus controls were mobilized into Agrobacterium GV3101 through the freeze-thaw method and transformed into wild-type *Arabidopsis thaliana* (Col-0) by floral dip (Clough and Bent, 1998). Selection of hygromycin resistant lines was described in Chapter 4.

GFP observation and GUS staining

GFP signals in 3-5 d old transgenic seedlings from ten independent T_2 lines were observed under either an epifluorescent or confocal microscope as described in Chapter 4. GUS staining of T_3 lines was conducted as described in Chapter 4. Representativ and consistent results were reported. All images were assembled in Adobe Photoshop CS (Adobe Systems).

Stress tolerance tests

Stress tolerance tests of mutants and 2-3 independet homozygous overexpression lines (T_3) compared to WT were performed according to Verslues *et al.* (2006) with modifications, which were reported in Chapter 4. The relative electrolyte leakage (REL) assay was performed according to Jiang *et al.* (2007). Each assay was repeated two to three times with similar results obtained. Data were analyzed using two-tailed

Student's *t*-test.

Microarray profiling, data analysis and validation

Qiagen Operon 26 k *Arabidopsis* oligonucleotide microarrays were used to profile transcriptomes of *wrky25-1* and *wrky33-1* T-DNA insertion mutants using WT as the reference. Total RNA samples from hydroponically cultured roots treated by 150 mM NaCl for 6 h with three independent biological replicates were extracted using the RNeasy Plant Mini kit (Qiagen). Possibly contaminating DNA was removed using the DNA-free kit (Ambion), and 5 μg of total RNA was reverse transcribed into cDNAs using RevertAid H- MMLV (Fermentas) and primers contained in the Array900 kit (Genisphere). Microarray hybridizations with dye-flip design were performed using the reagens contained in the Array900 kit according to the manufacturer's manual. Altogether six microarray slides were used with 12 image files analyzed. Data were analyzed as described in Jiang and Deyholos (2006).

To validate microarray data, qRT-PCR was performed with selected genes using the same batch of DNA-free RNA used for microarray hybridizations. The primers designed by PrimerExpress3.0 (Applied Biosystems) were: At5g05340 (*PER52*), Fwd, 5'-TCACCACGGCAGCTTCTTTC-3', Rvs, 5'-GTAGAGCCGCCGTTGAAGAG-3'; At5g24540 (glycosyl hydrolase family 1), Fwd, 5'-ACGGTTTGTTGCTCACGAT-3', Rvs, 5'-GTGTCGCCGGTACGATTTGT-3'; At4g23170 (protein kinase family protein), Fwd, 5'-AACGTCACGGTGGGACAAAC-3', Rvs, 5'-GAGAAGGCAACG CAGTTA-3'; At1g12610 (*DDF1/DREB1F*), Fwd, 5'-CGGAGGAACGGTGACAAA TG-3', Rvs, 5'-CACGCGCTGCCATATCTG-3'; At1g76680 (*OPR1*), Fwd, 5'-GGAA TGAGGCTGTGTCAAAGG-3', Rvs, 5'-GGTGCATCCACTTGGAACCT-3'; At2g46830 (*CCA1*), Fwd, 5'-GTCATCCCCACACAGTTGCA-3', Rvs, 5'-GCGAAA GTTGCGGCAGTATAA-3'; At3g14225 (*GLIP4*), Fwd, 5'-TGAGCTTGGGTCCATT TGGT-3', Rvs, 5'-TGGAGATTGATCAGCTCTGTGACT-3'; At4g25470 (*CBF2 /DREB1C*), Fwd, 5'-CCGGTTTCCTCAGGCGGTGATTACA-3', Rvs, 5'-TCCTCTG TAAATTGGGTGACG-3'; At3g62720, Fwd, 5'-TCCGAGAAGAAGCGGGTAAA-3', Rvs, 5'-TCCGTCGCCAGCAGATAAAC-3'; At2g34100, Fwd, 5'-TGTCGCGCG AGCAGAGAA-3', Rvs, 5'-GCAATTGTCTCTCGCCTCACA-3'; At4g12480, Fwd, 5'-GCACTGCGCTTAGGGCTAAC-3', Rvs, 5'-AAGCCAGACGGAACCTTTCTG-

3'; At4g10270, Fwd, 5'-GTGAGCATCGGAGCCGTAGA-3', Rvs, 5'-TAACGTTG TTCCGGAGATGTTG-3'; At5g10040, Fwd, 5'-GGAGGTTGGGTGGTTGATGA-3', Rvs, 5'-AGAAACCGCCACAGCAAACT-3'. qRT-PCR assay was performed using SYBR Green I fluorescent dye in ABI7500 fast real-time PCR machine as described in Chapter 4.

Analysis of promoter motifs in co-regulated gene sets.

The sequence of a 1,000-bp region immediately upstream (5′) of each gene's predicted translational start was scanned using the Motif Analysis program of TAIR (http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp). The frequency of all possible hexameric motifs in these 5′ regions was queried and compared with the frequencies of the same motifs in the total genomic set of 1000-bp 5′ regions. Motifs whose analysis yielded a P of the null hypothesis (no difference from the total genomic set) smaller than $1exp-4$ were deemed significant.

Sequence and phylogenetic analysis

The predicted protein sequences of 72 members of the *Arabidopsis* WRKY family were retrieved from the Database of *Arabidopsis* Transcription Factors (DATF, (http://datf.cbi.pku.edu.cn/) and Riken *Arabidopsis* Transcription Factors (RARTF, http://rarge.gsc.riken.jp/rartf/). The WRKY DNA binding domain boundary was defined as per Eulgem *et al* (2000) and only amino acid sequences from the single WRKY domain of group II and III members or the C-terminal WRKY domain of group I members was used, except WRKY19, for which, the N-terminal WRKY domain sequence was used because the C-terminal WRKY domain does not contain the typical WRKYGQK heptapeptide. Sequences were aligned according to Hall (2001) using ClustalX (v1.83) (Thompson *et al.*, 1997) with a gap opening penalty of 35 and gap extension penalty of 0.75 in pairwise alignment, and a gap opening penalty of 15 and gap extension penalty of 0.30 in multiple alignment parameters parameter settings. Multiple alignment was further checked with gaps manually inserted for optimal alignment. The parsimony tree was drawn using PAUP*4.0 (Sinauer Associates, Inc.). For Fig. 5-1, a postscript file (.ps) generated from ClustalX (v1.83) was read by Adobe Acrobat 7.0 professional with the PDF format file output.

Results

Identification and phylogenetic analysis of *WRKY25* and *WRKY33* genes

The majority of WRKY family TF genes are known to be responsive to biotic and/or abiotic stress, although most research has focused on the role of these genes in pathogen responses (Eulgem *et al.*, 2000). In our previously reported microarray data, we identified 18 WRKY TFs, including *WRKY25* (At2g30250) and *WRKY33* (At2g38470) that were induced by 150 mM NaCl treatment in *Arabidopsis* roots (Jiang and Deyholos 2006). This observation emphasizes a potentially important role for WRKY transcription factors in salt stress response. We chose to characterize *WRKY25* and *WRKY33* genes as their physiological role in abiotic stress has not been reported.

WRKY proteins are divided into three main classes based on sequence similarity, and additional structural motifs allow the distinction of different subgroups (Eulgem *et al.*, 2000). WRKY25 and WRKY33 are group Ia proteins, which contain two WRKY domains followed by a Cys2/His2-type zinc-finger motif (Fig. 5-1). *WRKY25* (At2g30250) encodes a protein of 393 amino acids with a molecular weight of 44.1 kD and an isolelectric point of 6.43, while *WRKY33* (At2g38470) encodes a protein of 519 amino acids with a molecular weight of 57.2 kD and an isolelectric point of 7.89 (Fig. 5-1). WRKY25 shares an overall identity of 28.90% at the amino acid level with the WRKY33 protein.

Phylogenetic analysis of genes is important for the functional analysis of a gene family. Members of transcription factor gene families usually contain a highly conserved domain or more than one conserved domain involved in DNA binding. However, sequence similarity beyond the domains is often relatively low. Further -more, a domain is considered as an evolutionary unit whose coding sequence can be duplicated and/or undergo recombination (Chothia *et al.*, 2003). Small proteins contain just one domain, whereas large proteins are formed by combinations of domains. As in the case of WRKY TFs, WRKY43 (At2g46130) is the shortest with only 93 aa, whereas WRKY19 (At4g12020) is the longest with 1798 aa. The main mechanisms of gene evolution are duplication, recombination, and sequence divergence (Ohno 1970; Chothia *et al.*, 2003). It was proposed that WRKY genes encoding a single WRKY domain may have been derived from the C-terminal WRKY domain of the genes

Fig. 5-1. Comparison of the deduced amino acid sequences of WRKY25 and WRKY33. The two WRKY motifs are underlined with the two cysteines and the two histidines of the zinc-finger motif indicated by arrows. The putative nuclear localization signal is shown by a double-headed arrow under the sequence. The asterisks above the sequence indicate identical amino acids, while symbols ':' and '.' indicate conserved and less conserved amino acids, respectively. PcWRKY1, parsley (*Petroselinum crispum*) WRKY1 (GenBank Acc# AAD55974).

harboring two WRKY domains (Wu *et al.*, 2005). We collected the protein sequences of all 72 *Arabidopsis* WRKY TFs and generated an inferred phylogenetic tree based on maximum parsimony analysis of the WRKY domain sequences (Fig. 5-2). *WRKY2, WRKY20, WRKY25, WRKY26* and *WRKY33* encode five structurally related WRKY proteins that belong to group Ia with each protein containing two highly conserved WRKY domains (Fig. 5-2).

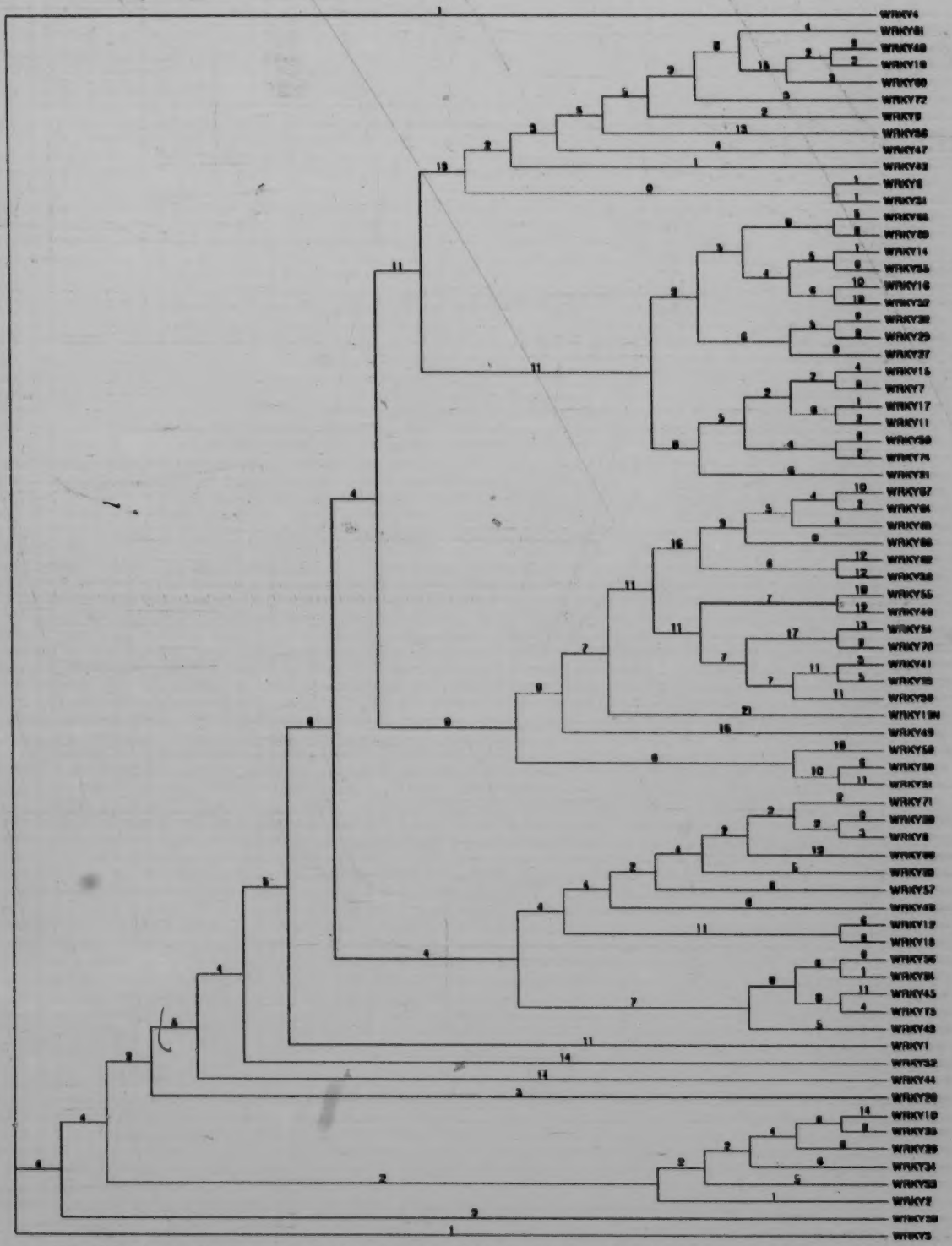


Fig. 5-2. A parsimony tree of 72 members of the *Arabidopsis* WRKY gene family. Only the amino acid sequences from the single WRKY domain of group II and III members or the C-

terminal WRKY domain of group I members were used in the tree construction using PAUP*4.0. The numbers on the branches represent branch length.

Our previous microarray data indicated that *WRKY2* and *WRKY26* were not especially responsive to NaCl treatment, while *WRKY20* transcripts increased in abundance by over 2-fold following 24 h and 48 h of exposure (Fig. 5-3). The transcript levels of *WRKY25* and *WRKY33* in *Arabidopsis* roots were greatly increased following salt treatment (Fig.5-3). Of the more than 70 *Arabidopsis* WRKY factors, WRKY33 is most similar to WRKY25 and to parsley WRKY1 involved in responses to pathogen elicitors (Andreasson *et al.*, 2005; Turck *et al*, 2004). Therefore, we focused on *WRKY25* and *WRKY33* in this study.

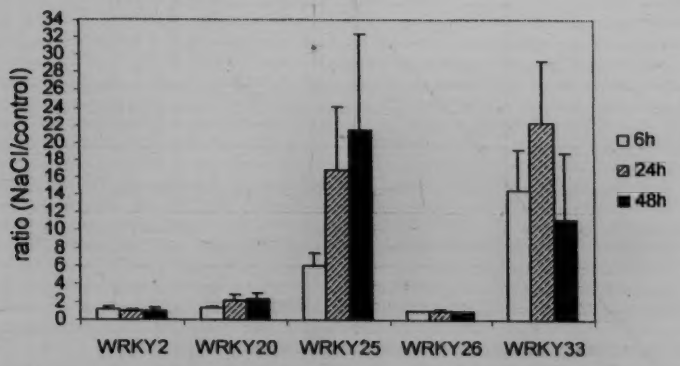


Fig. 5-3. Microarray results of *WRKY2, WRKY20, WRKY25, WRKY26* and *WRKY33* transcript abundance changes in response to salt stress. 20 DAS roots were treated by fresh ½ x MS or ½ x MS supplemented with 150 mM NaCl for 6, 24 or 48 h (Jiang and Deyholos, 2006). Data were from three biological replicates with S.D. shown as error bars.

Expression analysis of *WRKY25* and *WRKY33* transcripts in *Arabidopsis* tissues

To examine the expression patterns of *WRKY25* and *WRKY33* in different Arabidopsis tissues, we used qRT-PCR to detect their transcript levels in rosette leaves, roots, stem, flower buds, young siliques and dry seeds compared to their levels in roots. *WRKY25* mRNA was more abundant in roots than in any other tissues sampled. *WRKY33* was more abundant in roots as compared to leaves, stems, siliques, and seeds, however the transcripts were slightly more abundant in flowers than roots (Fig. 5-4 A, B).

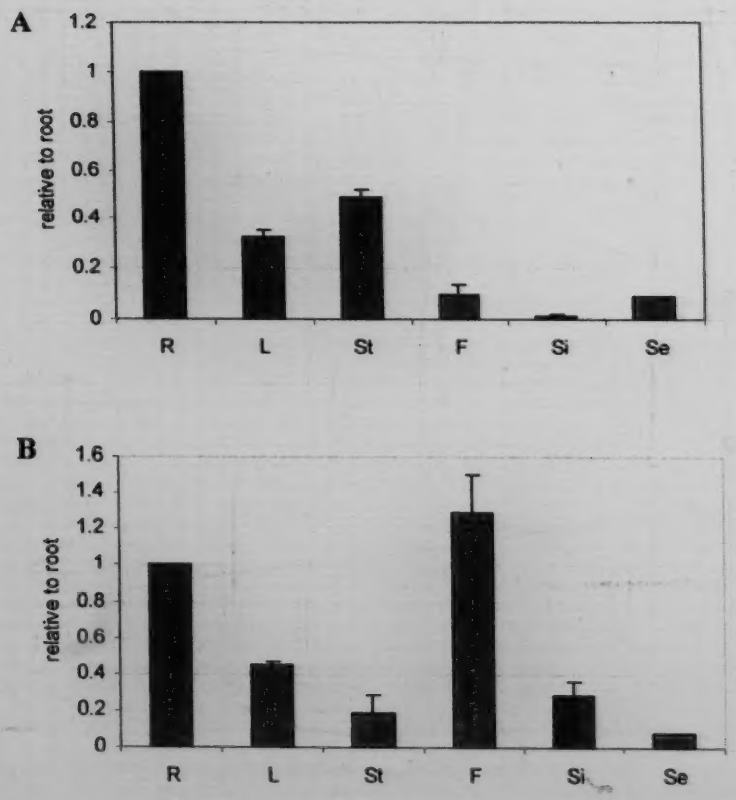


Fig. 5-4. qRT-PCR analysis of *WRKY25* (A) and *WRKY33* (B) transcript levels in various tissues and organs. R, roots; L, rosette leaves; St, stems; F, flower buds; Si, young siliques; Se, dry seeds. The abundance of mRNA in roots was assigned to be 1. Data were from three biological replicates with S.D. shown.

Subcellular localization

WRKY transcription factors are known to bind to cognate W-box *cis*-elements in the promoter regions of target genes to activate or repress their expression (Eulgem *et al.*, 2000; Rushton *et al.*, 1996). The deduced WRKY25 protein contains one nuclear localization signal (NLS), PEMKRMK (289-295), whereas the deduced WRKY33 protein contains two NLSs, PTKKKVE (215-221) and PEAKRWK (323-329) as predicted by PSORT (http://wolfpsort.org/, Horton *et al.*, 2006, Fig. 5-1). Additionally, WRKY33 protein is predicted to contain a 7-amino acid signal peptide, which may target WRKY33 to the chloroplast according to WoLF-PSORT and TargetP v1.1

(Emanuelsson *et al.*, 2000), as predicted by Schwacke *et al.* (2007). However, previous subcellular localization studies using GFP did not test if WRKY33 could be targeted to chloroplast, because either the 7 amino acid signal peptide was not included in the GFP fusion (Zheng *et al.*, 2006), or it simply was not investigated (Lippok *et al.*, 2007). To examine the sub-cellular localization of WRKY33, the coding regions was fused to the N-terminus of synthetic green fluorescent protein (sGFP) (Chiu *et al.*, 1996) and expressed constitutively under the control of cauliflower mosaic virus (CaMV) *35S* promoter. The WRKY33-sGFP fusion protein was found to be localized in the nucleus in root cells (Fig. 5-5A) and in the hypocotyl cells of transgenic seedlings harboring

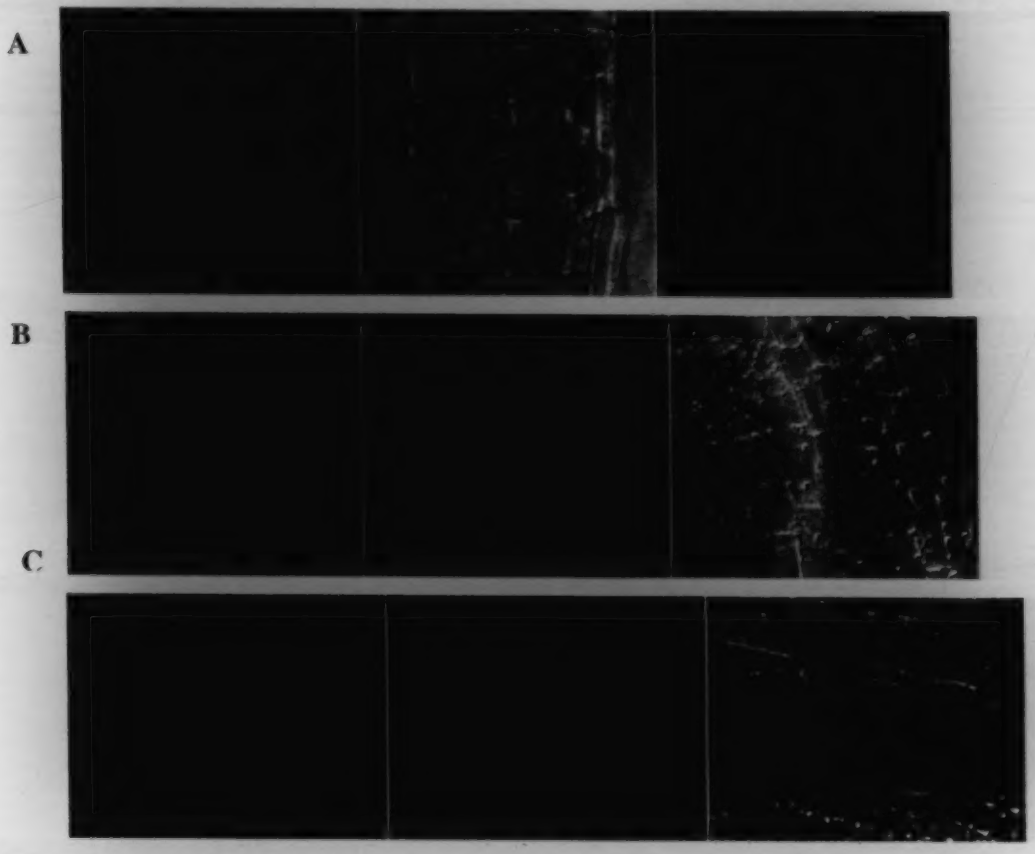


Fig. 5-5. Subcellular localization of WRKY33 fused to GFP in *Arabidopsis*.

A. Subcellular localization of WRKY33-sGFP in the root cells. The fluorescence (left) and bright-field (middle) images are overlaid at right.

B-C. Subcellular localization of WRKY33-sGFP (B) or ACS1-sGFP (C) in the hypocotyl cells. Left, chlorophyll autofluorescence; middle, GFP fluorescence; right, bright-field.

PWRKY33-sGFP, we failed to detect the fluorescence signal in the chloroplasts (Fig. 5-5B), which indicates that WRKY33 might not be targeted to chloroplasts except nuclei. As a positive control, subcellular of Arabidopsis cysteine synthase (*ACS1*, At2g43750, Hesse and Altmann, 1995) was also investigated. *ACS1* is a nuclear encoded gene with the protein being targeted to chloroplast. In hypocotyl cells, ACS1-GFP signal was observed in the chloroplasts only (Fig. 5-5C).

***WRKY25* and *WRKY33* are induced by multiple abiotic stresses**

Previous studies have shown that the expression of WRKY25 was elevated in cells in response to oxidative stress, heat shock, or wounding (Rizhsky *et al.*, 2004) and WRKY33 acts as a negative regulator of wound response/insect resistance genes and as a positive regulator of pathogen defense–related genes during JA signaling (Zheng *et al.*, 2006; Lippok *et al.*, 2007). To investigate the responses of *WRKY25* and *WRKY33* to various abiotic stresses or chemical treatments, qRT-PCR was used to compare the transcript levels in treated roots in relation to those in corresponding mocks or controls. Paraquat is a bipyridylium-type herbicide that generates superoxide anion inside cells.

The results showed that an increase in *WRKY25* transcript abundance was induced by salt at 6 or 24 h, which is consistent with our microarray results; and it was also induced by drought and Paraquat treatments at 6 h, or by mannitol, cold and heat at 24 h (Fig. 5-6A). *WRKY33* was induced by salt and cold at both time points, and it was also up-regulated by drought and ABA treatments at 6 h, and by osmotic stress at 24 h (Fig. 5-6B). We also monitored the changes of a stress marker gene *RD29A*, which was reported to be responsive to ABA, drought, cold, and salinity (Yamaguchi-Shinozaki and Shinozaki, 1993, 1994). Our qRT-PCR results confirmed that *RD29A* gene was induced by NaCl, cold, drought, ABA, and mannitol; and we also found that it was responsive to $AlCl_3$ treatment (Fig. 4-4B). On the whole, these results were consistent with the Affymetrix ATH1 results from the Genevestigator public microarray database (https://www.genevestigator.ethz.ch/at/) (data not shown).

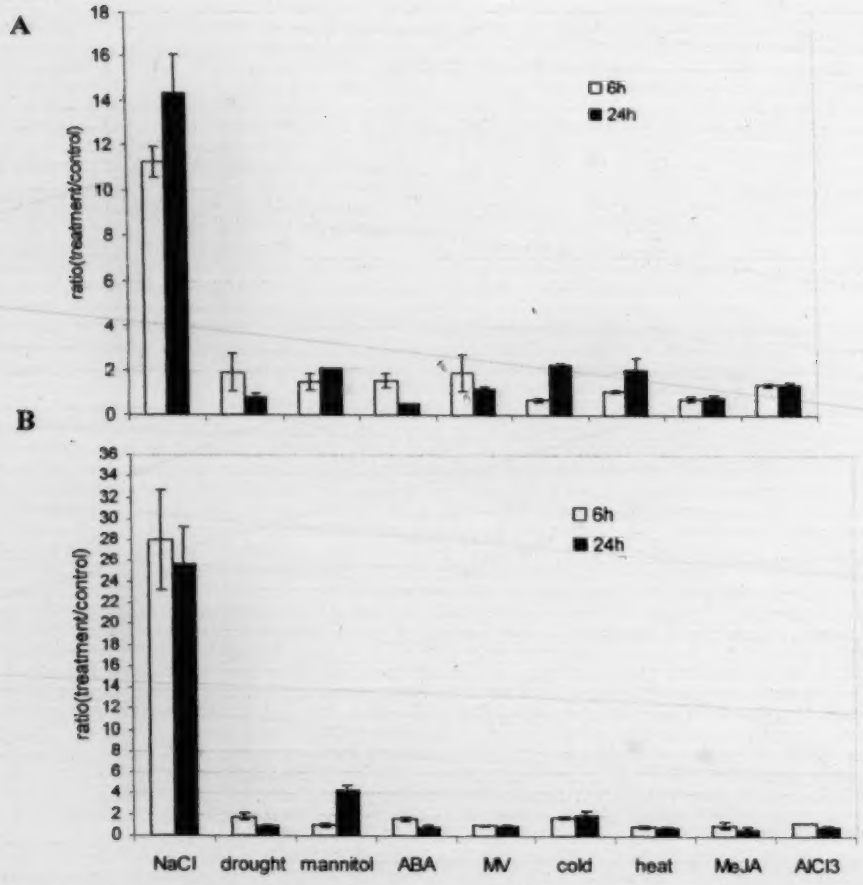


Fig. 5-6. qRT-PCR analysis of *WRKY25* (A) and *WRKY33* (B) transcript abundance in *Arabidopsis* roots subjected to various treatments as described in Materials and Methods. Values shown are the mean of three biological replicates and S.D. shown as error bars.

Expression analysis of *WRKY25* and *WRKY33* transcripts in ABA and SOS mutants

ABA is a phytohormone regulating several aspects of plant development including seed development, desiccation tolerance of seeds and seed dormancy, and plays a crucial role in the plant's response to abiotic stress (Finkelstein *et al.*, 2002). Stress-responsive genes have been proposed to be regulated by both ABA-dependent and ABA-independent signalling pathways (Chinnusamy *et al.*, 2005; Shinozaki and Yamaguchi-Shinozaki, 2006; Zhu, 2002). For example, RD29A works in both ABA-dependent and

ABA-independent signalling pathways while NaCl-induced *RD29B* accumulation requires ABA (Shinozaki and Yamaguchi-Shinozaki, 1997; Uno *et al.*, 2000).

Regulation of cellular ion homeostasis during salinity stress is critical for plant salt tolerance. Sodium extrusion from plant cells is mediated by the plasma membrane Na^+/H^+ antiporter SOS (Salt Overly Sensitive)1. Activation of SOS1 is controlled by SOS3 and SOS2. The *SOS3* gene encodes a myristoylated Ca^{2+} binding protein, which can interact with and activate the effector serine/threonine kinase, SOS2 (Zhu, 2002).

To examine whether the NaCl-induced accumulation of *WRK25* and *WRKY33* transcripts is dependent on the ABA or SOS pathways, we investigated the expression of these two genes in in ABA biosynthesis and signaling mutants, *aba1-5* and *abi4-1*,

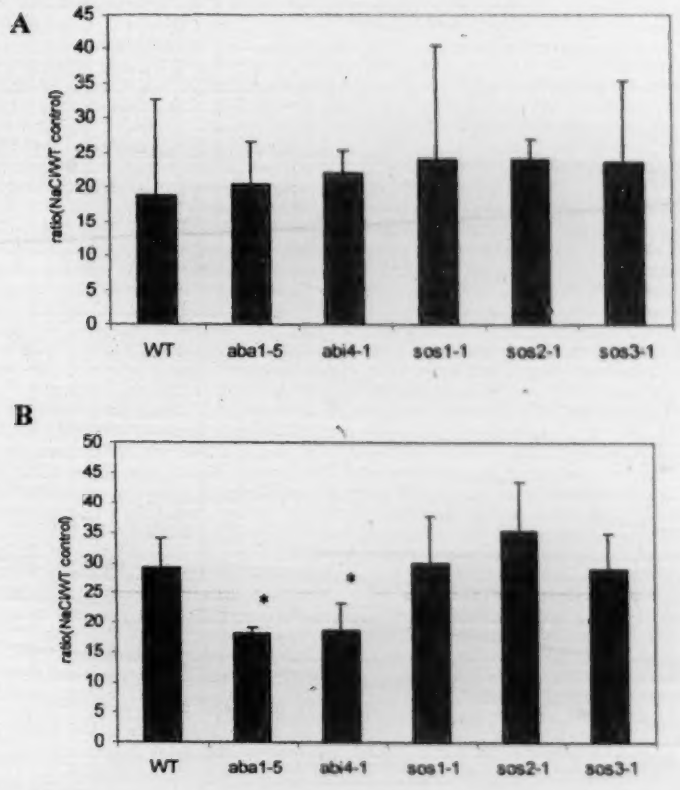


Fig. 5-7. qRT-PCR analysis of *WRKY25 (A)* and *WRKY33(B)* transcript abundance in wild-type (WT), *aba1-5, abi4-1, sos1-1, sos2-1*, and *sos3-1* mutants treated by 150 mM NaCl for 6 h. Asterisks indicate a significant difference ($p < 0.05$) between the mutant and WT plants. Data shown were the mean values from three biological replicates and error bars are the S.D.

and three SOS mutants, *sos1-1*, *sos2-1* and *sos3-1* using qRT-PCR. *aba1-5* is a mutant in which the zeaxanthin epoxidase (ZEP), is non-functional, while *abi4-1* is a mutation in a AP2 domain transcription factor responsible for ABA induced transcription (Finkelstein *et al.*, 2002). qRT-PCR results showed that transcript levels of *WRKY25* in any of the five mutants were not significantly different from WT (Fig. 5-7A), suggesting that salt-induced *WRKY25* expression is independent of ABA and SOS pathways. In the absence of normal ABA1 or ABI4 function, accumulation of *WRKY33* was reduced by 38% and 36%, respectively (Fig. 5-7B), therefore, *WRKY33* expression is partially dependent on ABA biosynthesis and signaling pathway. However, salt-mediated *WRKY33* induction was not influenced by any of the three *sos* mutants examined, indicating it is implicated in a SOS-independent pathway. As the *WRKY33* upstream fragment contained ABA responsive elements (ABRE), it was not surprising that salt-induced *WRKY33* accumulation was dependent on ABA.

Analysis of promoter-GUS fusion lines

To examine the role of the upstream regions of *WRKY25* and *WRKY33* genes on their expression in various *Arabidopsis* tissues, we fused a genomic fragment from immediately upstream of the ATG translational start codon (315 bp for PWRKY25 and 1 kb for PWRKY33 where P designates a fragment containing a putative promoter region) to a β-glucuronidase (GUS) reporter gene to determine their developmental, organ-specific and spatial expression patterns. We noted that the length of the intergenic sequence between *WRKY25* (At2g30250) and At2g30260 (encodes U2 small nuclear ribonucleoprotein B) is only 203 bp, so we cloned this intergenic sequence plus the 5'UTR of At2g30250 into our reporter gene fusion.

To avoid potential problems with CaMV35s enhancers, we cloned the promoter regions into a modified binary vector, p0381Z, which did not contain the CaMV35S promoter or a plant selectable marker gene. We therefore co-transformed these promoter-GUS constructs into *Arabidopsis* along with another binary vector (pCAMBIA1300) which provided a selectable marker but not a reporter fusion, and which presumably integrated into the plant genome independently of our reporter fusions.

We examined ten independent T_3 transgenic *Arabidopsis* lines to determine the

GUS expression patterns conferred by the *WRKY25* and *WRKY33* upstream regions. For the PWRKY25-GUS fusion, in 5 d old seedlings, moderate GUS expression was visible in the hypocotyl, root, as well as at the tip of the cotyledon (Fig. 5-8a). In 18 d old seedlings, GUS expression was evident in the leaf veins and roots (Fig. 5-8b). In inflorescence, GUS expression is only visible in stamens, weak in petals (Fig. 5-8c). In young siliques, no GUS expression was observed (Fig. 5-8d).

For plants bearing PWRKY33-GUS fusions, in 5 d old, untreated seedlings, moderate GUS expression was visible in the leaf veins, and less intensely in roots (Fig. 5-8e). In 18 d old seedlings, GUS expression was also evident both in the leaf veins and in the roots (Fig. 5-8f). In inflorescences, GUS expression was only visible in petals and sepals (Fig. 5-8g). In young siliques, weak GUS expression was observed in the tip and restricted area of the silique (Fig. 5-8h).

We then examined the GUS expression in 19 d old seedlings treated by moderate salt, drought, ABA or JA for 6 h and compared the staining pattern to GUS expression in untreated leaves from the same transgenic lines. After 150 mM NaCl and 10 μM MeJA treatments, we observed a stronger GUS staining in both roots and leaves of PWRKY25-GUS lines (Fig. 5-8j, k). We also observed stronger GUS staining in both roots and leaves of PWRKY33-GUS lines after 150 mM NaCl and dehydration treatments, (Fig. 5-8m, n). Our results demonstrated that the upstream sequence present in PWRKY25-GUS contains sufficient cis-elements to respond to salt or JA treatments, while the upstream fragment present in PWRKY33-GUS contains sufficient cis-elements to respond to salt or drought treatments.

We analyzed the cis-elements contained in the promoter regions of *WRKY25* and *WRKY33* genes using PlantCare (Lescot *et al*., 2002; Table 5-1). Only one MeJA-, one GA- and one heat stress responsive elements were identified in the 315 bp upstream region of WRKY25. However, the 1 kb upstream region of *WRKY33* contained heat, ABA, wounding, SA and low temperature responsive elements, indicating that *WRKY33* may respond to these treatments. Interestingly, *WRKY33* promoter region also contained two W-box elements, suggesting its activity might be regulated by another WRKY TF.

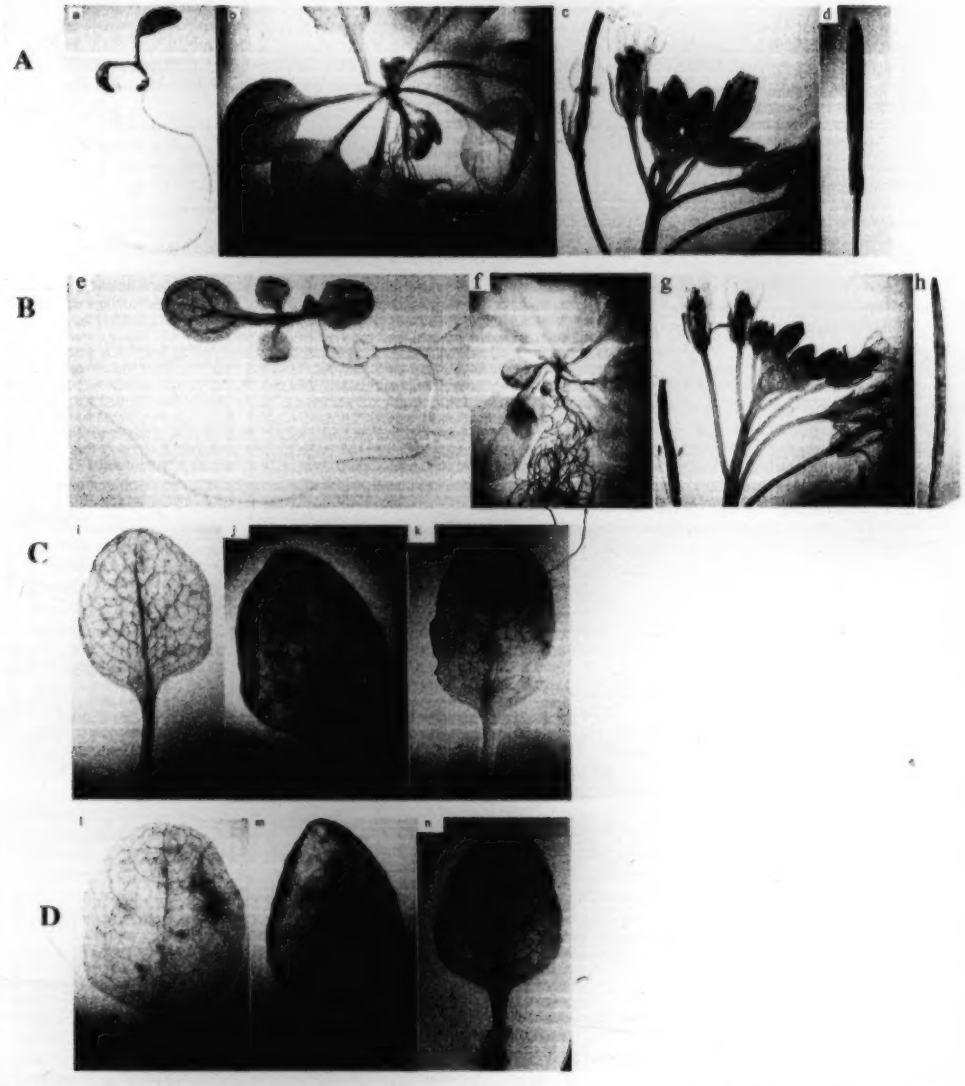


Fig. 5-8. Histochemical localization of GUS activity in representative transgenic *Arabidopsis* plants harboring promoter-GUS constructs.

A-B, Histochemical localization of GUS activity in representative transgenic plants harboring PWRKY25-GUS (A) or PWRKY33-GUS (B). a &e, GUS staining pattern in 5 d old seedling; b&f, GUS staining pattern in 18 d old seedling; c&g, GUS staining pattern in inflorescence; d&h, GUS staining pattern in 7 DAF old siliques.

C, GUS staining pattern of transgenic lines harboring PWRKY25-GUS in 19 d old leaves untreated (i) or treated with 150 mM NaCl (j) or 10 μM JA (k) for 6 h.

D, GUS staining pattern of transgenic lines harboring PWRKY33-GUS in 19 d old leaves untreated (l) or treated with 150 mM NaCl (m) or drought (n) for 6 h.

Table 5-1. Putative regulatory sequences found in the promoter regions of *WRKY25* and *WRKY33*.

motif	sequence	position	strand	function of regulatory elements
WRKY25 promoter				
GARE-motif	AAACAGA	-38	-	gibberellin-responsive element
HSE	AAAAAATTTC	-61	+	heat stress responsiveness
TGACG-motif	TGACG	-308	+	MeJA-responsiveness
WRKY33 promoter				
HSE	AAAAAATTTC	-509	-	heat stress responsiveness
ABRE	TACGTG	-412	+	abscisic acid responsiveness
ARE	TGGTTT	-694	-	essential for the anaerobic induction
AT-rich sequence	TAAAATACT	-624	+	maximal elicitor-mediated activation (2 copies)
W box	TTGACC	-868	-	wounding and pathogen response
		-270	-	
O2-site	GATGACATGA	-581	-	zein metabolism regulation
TC-rich repeats	ATTTTCTTCA	-70	+	defense and stress responsiveness
TCA-element	GAGAAGAATA	-66	-	salicylic acid responsiveness
CCAAT-box	CAACGG	-274	+	MYBHv1 binding site
LTR	CCGAAA	-515	+	low-temperature responsiveness
MBS	CGGTCA	-271	+	MYB Binding Site

The 315 bp and 1 kb sequences upstream of ATG start codon of *WRKY25* and *WRKY33*, respectilvey were analyzed using PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Putative elements involved in light responses (found in all promoters) and the core promoter element (e.e. TATA-box) are not included in the table. The nucleotide immediately before ATG start codon is designated -1.

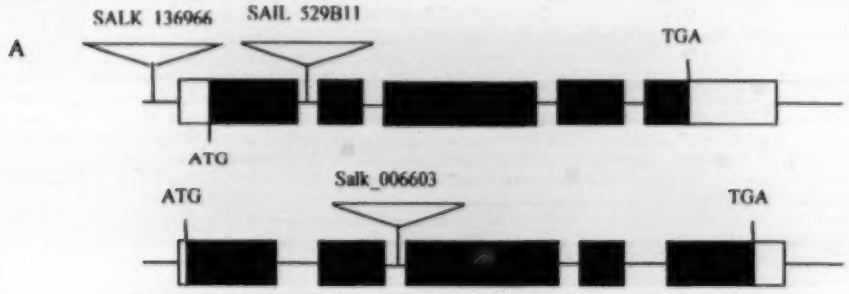
Mutant phenotypes of *WRKY25* and *WRKY33*

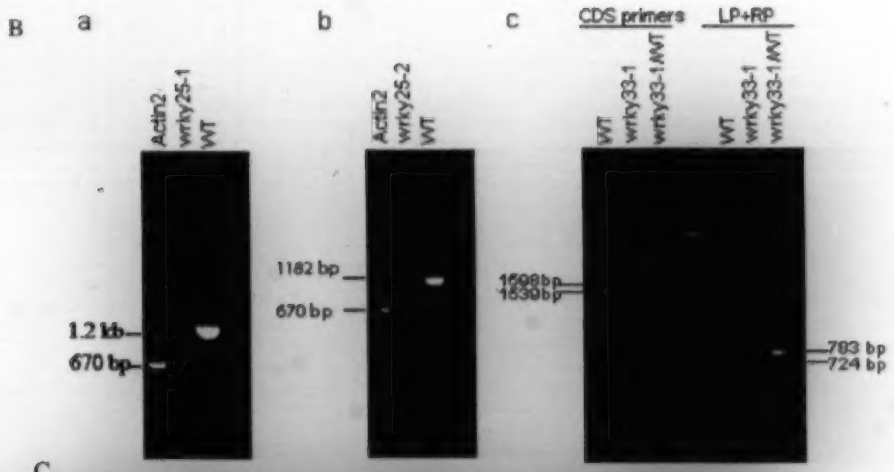
In order to investigate the function of *WRKY25* and *WRKY33* in abiotic stress, we analyzed their mutant phenotypes. We obtained two T-DNA insertion mutants for *WRKY25*, designated as *wrky25-1* (SAIL_529B11) and *wrky25-2*(SALK_136966), and one for *WRKY33*, designated as *wrky33-1* (SALK_006603) (Fig. 5-9A). We used PCR and RT-PCR to confirm homozygosity of these mutant alleles and absence of detectable wild-type transcripts for the mutated genes (Fig. 5-9B). In *wrky25-2*, we

detected a partly reduced *WRKY25* transcript abundance (Fig. 5-9B), so we used only *wrky25-1* for the following experiments. In SALK_006603, we detected a PCR product that was longer than the corresponding band from WT (Fig.5-9B), and after cloning and sequencing, concluded that this line contained a T-DNA induced chromosomal rearrangement. This allele contained no T-DNA insertion but had a 33 bp deletion followed by a 23 bp duplication at the 3'-end of the second intron of WRKY33 that would prevent proper splicing; therefore, a larger transcript with an unspliced second intron would direct the translation of a truncated protein of 209 amino acids containing no WRKY zinc-finger motif, as reported by Zheng *et al.*(2006).

Under normal growth conditions, we did not observe any morphological differences between any of the confirmed null mutants and WT plants grown in soil. Root elongation and root bending assays of the mutants (compared to WT) were conducted under 100 or 150 mM NaCl or mannitol, 0.5 μM methyl viologen (Paraquat), and 0.5 μM ABA treatments. We found that *wrky25-1* mutants did not show significant difference compared to WT plants under all the stress conditions tested (Fig. 5-9C, F), while *wrky33* mutant was more sensitive to salt stress (Fig. 5-9D, G).

Considering the functional redundance between members of WRKY gene family, especially between members belonging to the same group in the phylogenetic tree (Fig. 5-2), we crossed *wrky25-1* to *wrky33-1* and screened out homozygous double mutant *wrky25wrky33* by PCR and confirmed its homozygosity by RT-PCR. Similarly, we performed root elongation test on ½ x MS medium plates containing 100 mM NaCl. We observed that *wrky25wrky33* mutant roots were significantly shorter than WT




B
a
b
c
CDS primers
LP+RP
Actin2
wrky25-1
WT
Actin2
wrky25-2
WT
WT
wrky33-1
wrky33-1/WT
WT
wrky33-1
wrky33-1/WT
1182 bp
670 bp
1.2 kb
670 bp
1698bp
1639bp
783 bp
724 bp

C

D

E

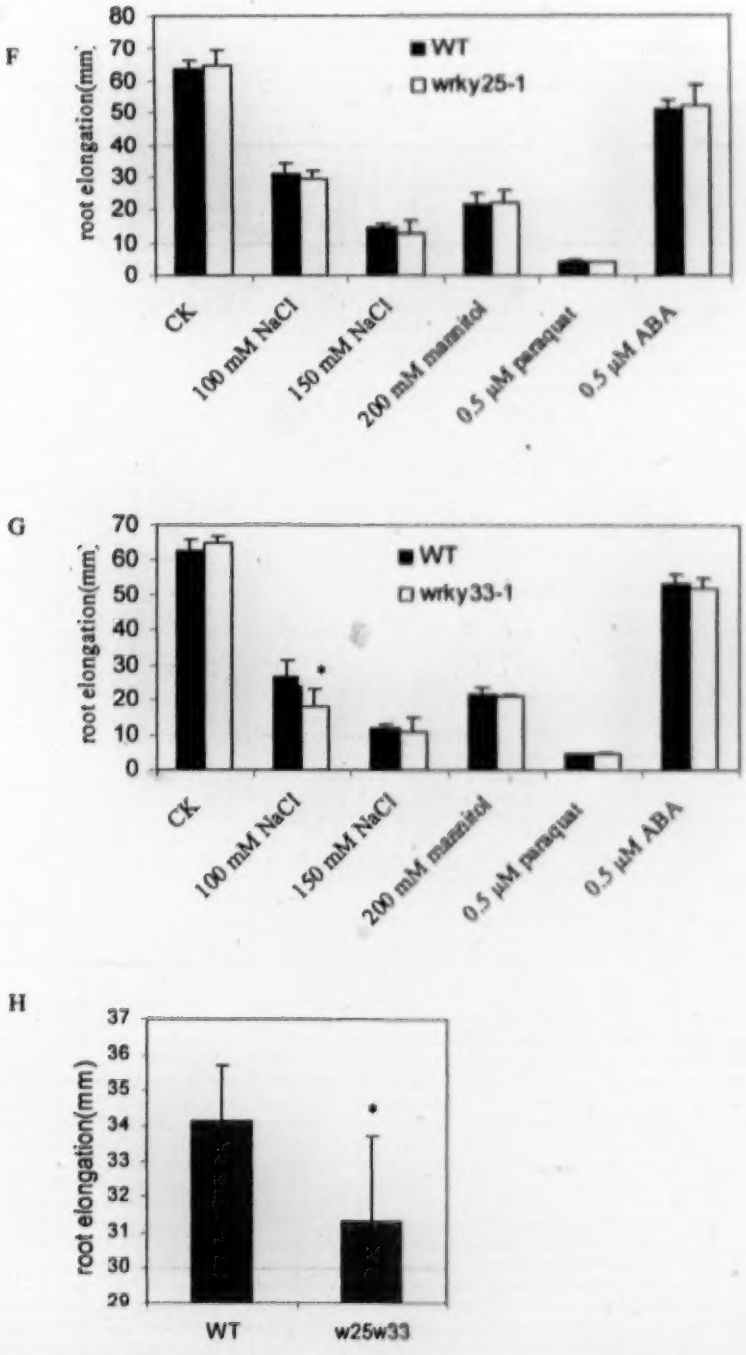


Fig. 5-9. Identification of T-DNA insertion mutants of *WRKY25* and *WRKY33* genes and stress tolerance assay.

A. Schematic diagrams of *WRKY25* and *WRKY33* genes and T-DNA insertion sites. Closed

boxes represent exons (black, coding regions; empty, 5'UTR or 3'UTR), and lines between closed boxes represent introns.

B. RT-PCR validation of homozygous mutant lines of *wrky25-1*(a), *wrky25-2*(b) and *wrky33-1*(c) using the coding region (CDS) primers and *ACTIN2* gene primers as a positive control. WT, wild-type (Col-0) cDNAs. For SALK_006603, the LP/RP primers were the same as those used for PCR screening (see "Materials and Methods" section).

C, Stress tolerance test of *wrky25-1* (SAIL_529_B11) mutants on ½ x MS containing 100 or 150 mM NaCl, 200 mM mannitol, 0.5 μM MV, or 0.5 μM ABA.

D, Stress tolerance test of *wrky33-1* (SALK_006603) mutants on ½ x MS containing 100 mM NaCl, 200 mM mannitol, 0.5 μM MV, or 0.5 μM ABA.

E, Stress tolerance test of *wrky25wrky33* double mutants on ½ x MS containing 100 mM NaCl.

F. Statistical analysis of the root elongation assay of *wrky25-1*mutants.

G. Statistical analysis of the root elongation assay of *wrky33-1*mutants.

H. Statistical analysis of the root elongation assay of *wrky25wrky33* mutants. Data are means ± S.D. Asterisks indicate a significant difference ($p < 0.05$) between the mutant and WT exposed to the same treatment on the same plate.

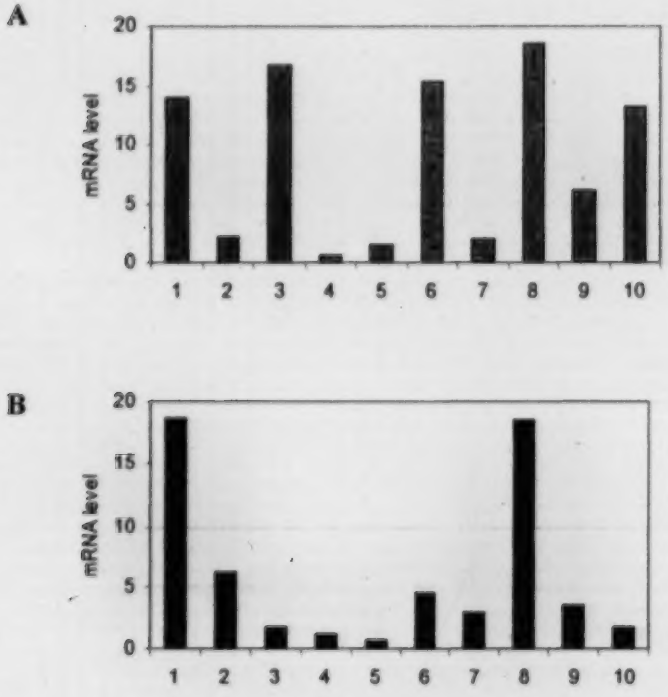
($p<0.01$), suggesting that *wrky25wrky33* double mutant was more sensitive to salt (Fig. 5-9E, H). The performance of *wrky25wrky33* under other types of abiotic stress conditions has not been tested yet.

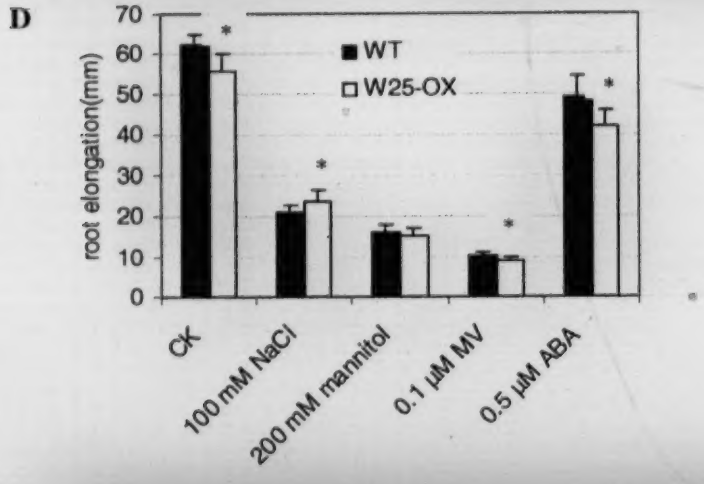
Overexpression of *WRKY25* and *WRKY33* genes in Arabidopsis

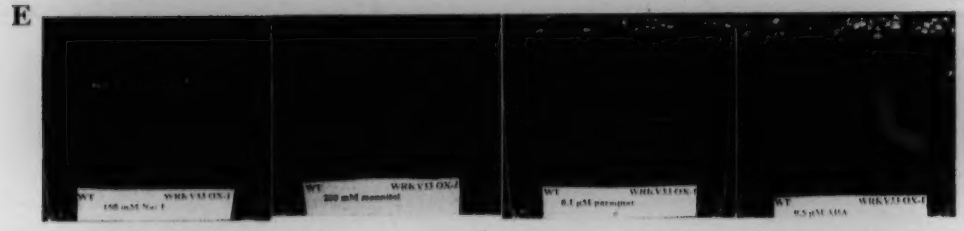
The putative roles of WRKY25 and WRKY33 in abiotic stress were further examined by overexpressing them in wild-type Arabidopsis. To do so, we cloned and fused their coding regions under the control of the CaMV35S promoter in Arabidopsis plants. None of the resulting transgenic plants showed detectable phenotypic changes compared with WT plants grown under the same, non-stressed conditions. The expression level of each transgene was evaluated for 10 independent T_2 lines by qRT-PCR. We chose 2-3 lines showing the highest transgene expression for each of the two WRKY genes for further characterization. For WRKY25, lines 3, 6 and 8 (Fig. 5-10A), and for WRKY33, lines 1 and 8 (Fig. 5-10B).

We subjected the selected transgenic lines to various abiotic stress treatments, NaCl, mannitol, Paraquat, ABA, cold and heat, and quantified the root elongation in

comparison to WT seedlings. Roots of *WRKY25*-overexpressing plants grew longer (8%) than that of WT under 100 mM NaCl (Fig 4-10C, D), and they were also more sensitive to Paraquat and ABA treatments as root elongation was inhibited by Paraquat and leaves showed senescence compared to those of WT (Fig 4-10C, D). The lateral roots of *WRKY25*-overexpressing plants were fewer than those of WT under the mannitol treatment, although root elongation was not influenced (Fig. 5-10C, D). Overexpression of *WRKY33* in *Arabidopsis* enhanced the tolerance of transgenic plants to salt stress, as 40% of WT seedling leaves were bleached by 150 mM NaCl, while almost no overexpressing line was bleached by salt treatment (Fig. 5-10E, F). However, similar to *WRKY25*-overexpressing lines, *WRKY33*-overexpressing plants were also more sensitive to Paraquat and ABA treatments than WT as shown by leaf senescence under ABA and significantly shorter roots under moderate ABA treatment ($p<0.05$, Fig. 5-10E, F). Under Paraquat treatment, root elongation of *WRKY33*-overexrpessing lines were significantly shorter than WT, and the leaves of *WRKY33* transgenic lines showed senescence with fewer lateral roots than those of WT plants (Fig. 5-10E, F).







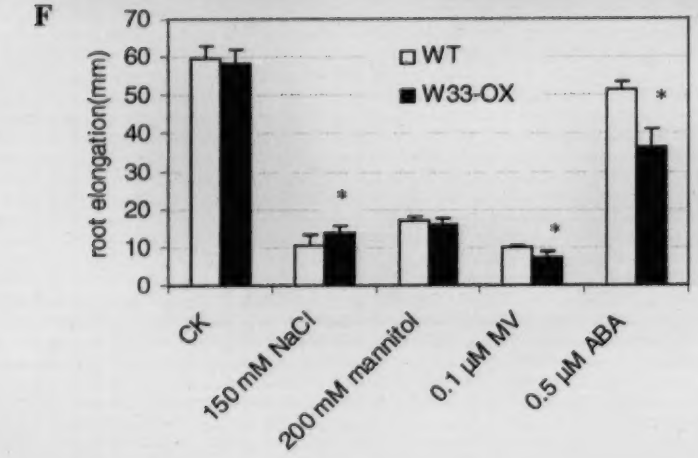


Fig. 5-10. Identification and stress tolerance testing of *WRKY25*- and *WRKY33*-overexpressing

Arabidopsis plants.

A-B, qRT-PCR analyses of transcript levels of *WRKY25* and *WRKY33* in 10 independent transgenic lines (T_2). Rosette leaves harvested from 28 d old plants were used for the assay. The ratios were that of genes in overexpressed lines compared to that of in wild-type and normalized to the level of endogenous gene *UBQ10*.

C, E. Performance of *WRKY25*-(C) and *WRKY33*-overexpressing (E) transgenic plants and wild-type plants under salt, mannitol, paraquat or ABA treatments.

D-F. Effect of 100 or 150 mM NaCl, 200 mM mannitol, 0.1 μM Paraquat or 0.5 μM (±)-ABA on root elongation of WT and *WRKY25*- or *WRKY33*-overexpressing seedlings. Data are means ± S.D. (n = 8–11). Asterisks indicate a significant difference ($p < 0.05$) between the overexpressing plants (OX) and WT exposed to the same treatment.

Microarray identification of target genes of WRKY25 and WRKY33

To identify genes that had different expression patterns in the absence of WRKY25 or WRKY33 as compared to WT, we used full-genome oligonucleotide microarrays representing 26, 090 genes. RNA was extracted from *wrky25-1*, *wrky33-1* mutants or WT plants, where each of these three genotypes had been treated with 150 mM NaCl for 6 h. Differences in the expression of genes were analyzed by SAM with a FDR of ~5%. After applying a 1.5-fold cutoff, only 16 and 15 genes were down- and up-regulated, respectively, in *wrky25-1* mutant compared to WT (Tables 5-2 & 5-3). In contrast, we detected, using a 1.5-fold cutoff, 87 up-regulated genes and 121 down-regulated genes in *wrky33-1*mutants (Tables 5-4 & 5-5).

The genes that showed different profiles between the *wrky33* mutant and the wild type were classified into 10 categories (Tables 5-4 & 5-5). We observed that the down-regulated genes are quite distinct from the up-regulated genes. For instance, several types of transcription factor, such as AP2/ERF, AUX/IAA, MYB, and zinc-finger family members, were induced in the absence of *wrky33* (Table 5-4). In contrast, quite a few members of class III peroxidase family, glycosyl hydrolase family, and glutathione transferase (GST) family were down-regulated in *wrky33* (Table 5-5).

In the WRKY25 regulon, three protease inhibitor/seed storage/lipid transfer protein (LTP) genes were found to be up-regulated (Table 5-2). LTPs form a multigenic family and can inhibit the growth of fungal pathogens *in vitro* and are also

Table 5-2. Genes that are up-regulated by 150 mM NaCl in *wrky25-1* mutant.

AGI	Putative identity	F.C.	S.D.	W-box	W-box-like
At2g34100	unknown protein, similar to diacylglycerol kinase eta [Strongylocentrotus purpuratus]	37.2	15.00	+	+
At3g62720	Xyloglucan xylosyltransferase(ATXT1)	11.84	6.32	+	+
At4g12480	protease inhibitor/seed storage/lipid transfer protein (LTP) family	2.2	0.81	-	+
At4g12470	protease inhibitor/seed storage/lipid transfer protein (LTP) family	1.8	1.02	-	+
At4g22470	protease inhibitor/seed storage/lipid transfer protein (LTP) family	1.63	0.11	+	+
At2g33780	VQ motif-containing protein contains PF05678: VQ motif	1.78	0.86	+	-
At5g65140	trehalose-6-phosphate phosphatase, putative	1.77	0.61	-	+
At4g23590	aminotransferase family	1.71	0.67	+	+
At3g27360	histone H3	1.7	0.64	+	+
At1g56150	auxin-induced protein family	1.63	0.22	+	+
At5g57990	ubiquitin-specific protease 23 (UBP23), putative	1.55	0.65	+	-
At1g10880	Unknown protein	1.55	0.25	+	-
At5g26260	meprin and TRAF homology domain-containing protein	1.54	0.09	+	+
At5g47440	hypothetical protein	1.54	0.48	+	-
At2g36800	DON-Glucosyltransferase(DOGT1)	1.53	0.43	+	+

AGI, *Arabidopsis* genome initiative; fold change (F.C.) is expressed as the mean value the mRNA level of mutant/WT from three biological replicates; S.D. indicates the standard deviation. The presence or absence of W-box or W-box-like motif in the 1 kb upstream regions is represented by + or -, respectively.

involved in the signalling of defence mechanism of plants against their pathogens (Blein *et al.*, 2002). However, the exact function of LTPs in abiotic stress is not well understood. We also detected *ATXT1*, which is involved in plant cell wall modification (Cavalier and Keegstra, 2006) and *DOGT1* implicated in detoxification of the fusarium mycotoxin deoxynivalenol (Poppenberger *et al.*, 2003) in the up-regulated gene list (Table 5-2).

Among the down-regulated genes in absence of *WRKY25*, there are two pyruvate decarboxylase (PDC) genes working in fermentation, an acyl-(acyl-carrier-protein) desaturase gene for fatty acid biosynthesis, and one sucrose synthase gene (Table 5-3).

Table 5-3. Genes that are down-regulated by 150 mM NaCl in *wrky25-1* mutant.

AGI	Putative identity	F.C.	S.D.	W-box	W-box-like
At5g54960	pyruvate decarboxylase (PDC2)	0.67	0.17	-	+
At4g33070	pyruvate decarboxylase-1 (PDC1)	0.59	0.15	+	-
At5g58070	lipocalin, temperature stress-induced	0.66	0.19	+	+
At2g16060	class 1 non-symbiotic hemoglobin (AHB1/GLB1)	0.65	0.19	+	-
At4g30270	xyloglucan endotransglycosylase (MERI5B)	0.65	0.24	+	-
At5g15120	expressed protein	0.64	0.19	+	+
At1g19530	expressed protein	0.62	0.26	+	+
At2g28550	AP2 domain transcription factor RAP2.7/TOE1	0.62	0.29	+	+
At1g43800	acyl-[acyl-carrier-protein] desaturase (stearoyl-ACP desaturase), putative	0.6	0.25	+	+
At5g39890	expressed protein	0.6	0.26	+	+
At3g03270	universal stress protein (USP) family protein / early nodulin ENOD18 family protein	0.58	0.25	-	+
At3g43190	sucrose synthase (UDP-glucose-fructose glucosyltransferase/sucrose-UDP glucosyltransferase), putative	0.57	0.23	+	+
At1g33055	expressed protein	0.52	0.19	+	-
At3g02550	lateral organ boundaries (LOB) domain protein 41 (LBD41)	0.52	0.20	+	-
At5g10040	expressed protein	0.51	0.10	+	+
At4g10270	probable wound-induced protein	0.47	0.13	-	+

In addition, one cell wall-modifying gene, *MERI5B*, and, one AP2 TF (RAP2.7 /TOE1) involved in miRNA-mediated flowering time control (Aukerman and Sakai, 2003) and, one hemoglobin gene shown to be able to protect plants from oxidative stress (Yang *et al.*, 2005).

In the WRKY33 regulon, we detected 208 differentailly expressed genes belonging to 10 functional categories (Tables 5-4 and 5-5). Severn members of the AP2/ERF family including two of the best characterized stress-responsive transcription factors, CBF1/DREB1B and CBF2/DREB1C (Stockinger EJ *et al.*, 1997; Gilmour SJ *et al.*, 1998; Medina J *et al.*, 1999) were up-regulated (Table 5-4). Another AP2/ERF family gene (*DDF1/DREB1F*) was previously found to be induced by high salinity and is involved in the regulation of GA biosynthesis and stress tolerance (Magome *et al.*, 2004). Three auxin induced genes (*IAA17, IAA13,* and *IAA3*) were up-regulated in *wrky33* mutant. These three IAAs were found to be involved in root development and

light response (Tian and Reed, 1999; Liscum and Reed, 2002). These results indicate that salt stress response and auxin signalling may also interact with each other. We also detected three MYB-related TFs, comprising a single MYB repeat instead of two or three as in most MYB proteins. Among these, CCA1 was found to regulate circadian rhythms (Wang and Tobin 1998). Furthermore, five zinc-finger family members were found to be up-regulated in the absence of *WRKY33*, but their function is largely unknown except COL2, which is implicated in flower development (Ledger *et al.*, 2001). Two genes (*OPR1* and *OPR2*) involved in jasmonic acid biosynthesis were also up-regulated under salt stress in *wrky33*. Both *OPR1* and *OPR2* were up-regulated by senescence, JA and salicylic acid, which confirmed their involvement in the modulation of JA- or SA- responsive gene expression and disease resistance (Eulgem and Somssich 2007). The discovery of up-regulation of OPR1 and OPR2 in *wrky33* suggests that WRKY33 is a negative regulator of JA signaling.

Interestingly, seven members of class III peroxidase gene family were down-regulated in *wrky33* (Table 5-5). Class III peroxidases are plant-specific oxidoreductases that are implicated in various physiological processes such as H_2O_2 detoxification, auxin catabolism, liginfication, suberization, stress response (wounding, pathogen attack, NaCl) and senescence (Hiraga *et al.*, 2001). We previously reported that the majority of class III peroxidases are responsive, at the transcript level, to NaCl treatment in *Arabidopsis* roots (Jiang and Deyholos, 2006). The specific functions of these genes remain to be determined. Three glutathione-S-transferases (GSTs) genes, all belonging to Tau sub-family, were also repressed in *wrky33* (Table 5-4). GSTs are proposed to play a role in oxidative stress and apoptosis (Kilili *et al.*, 2004; Apel and Hirt 2004). Although these genes were induced in salt-treated WT plants, they were down-regulated in the *wrky33* mutant, suggesting that WRKY33 acts upstream of these three GST genes. Five glycosyl hydrolase family genes, were also identified to be down-regulated in *wrky33* mutant (Table 5-5). Glycosyl hydrolases play a role in cell wall remodeling (Xu *et al.*, 2004).

We identified one *WRKY* gene, *WRKY28*, that was down-regulated by salt in *wrky33* (Table 5-5). In WT plant roots, *WRKY28* was strongly induced by salt treatment (Jiang and Deyholos, 2006). Further examination showed that the *WRKY28*

Table 5-4. Genes that are positively regulated by 150 mM NaCl in *wrky33-1* mutant.

AGI	Putative identity	F.C.	S.D.	W-box	W-box-like
Cell growth and division					
At2g42070	MutT/nudix family protein	1.67	0.34	+	+
At3g53830	regulator of chromosome condensation (RCC1) family protein	1.5	0.25	+	-
Cell rescue, defense, death and aging					
At1g59860	heat shock protein, putative	1.52	0.34	+	+
At1g66090	disease resistance protein (TIR-NBS class), putative	1.51	0.4	+	-
Cellular communication/Signal transduction			0		
At3g62260	protein phosphatase 2C (PP2C), putative	1.72	0.36	+	+
At3g10190	calmodulin, putative	1.56	0.16	+	-
At1g73800	calmodulin-binding protein	1.62	0.46	+	-
Cellular structure, organization and biogenesis					
At2g28950	expansin, putative (EXP6)	1.6	0.26	-	+
At4g33930	glycine-rich protein	1.76	0.31	-	+
At2g36800	putative glucosyl transferase	1.8	0.72	+	+
At5g67230	glycosyltransferase family 43 protein	1.52	0.32	+	+
At1g23760	polygalacuronase isoenzyme 1 beta subunit - related	1.64	0.28	+	-
Cellular transport and transport facilitation					
At1g29100	copper-binding protein family	1.57	0.32	+	+
At3g44190	pyridine nucleotide-disulphide oxidoreductase family	1.67	0.34	-	-
At3g17690	cyclic nucleotide-binding transporter 2 (CNBT2/CNGC19)	1.71	0.44	-	-
At2g13790	leucine-rich repeat protein kinase family	1.51	0.39	+	+
At3g28740	cytochrome P450 family	1.51	0.17	-	+
At1g50590	pirin-like protein	1.65	0.25	+	+
At3g04620	nucleic acid binding	1.59	0.43	+	+
At1g04570	integral membrane transporter family protein	1.51	0.28	-	-
At5g51980	nucleic acid binding; similar to ZFWD1 (zinc finger WD40 repeat protein 1)	1.66	0.34	+	+
Metabolism					
At4g31780	1,2-diacylglycerol 3-beta-galactosyltransferase (UDP-galactose:diacylglycerol galactosyltransferase) (MGDG synthase) (MGD1), putative	1.51	0.32	+	-
At5g14740	CARBONIC ANHYDRASE 2	1.59	0.43	+	+
At5g22140	pyridine nucleotide-disulphide oxidoreductase family	1.51	0.18	-	+
At4g20320	CTP synthase (UTP--ammonia ligase), putative	1.64	0.37	+	-
At1g76680	12-oxophytodienoate reductase (OPR1)	2.12	0.89	+	-
At1g76690	12-oxophytodienoate reductase (OPR2)	1.87	0.54	+	+

Protein fate (folding, modification, and destination)					
At5g56010	heat shock protein, putative	1.56	0.12	+	-
At2g22920	serine carboxypeptidase -related	1.64	0.17	+	-
At5g10540	peptidase M3 family protein / thimet oligopeptidase family protein	1.6	0.37	+	+
At4g23570	phosphatase – related(SGT1A)	1.57	0.21	+	+
At5g51450	RING-finger type ubiquitin ligases(RIN3)	1.92	0.82	+	-
Transcription					
At1g12610	AP2/ERF transcription factor, putative(DREB subfamily)/dwarf and delayed-flowering1 (DDF1/DREB1F)	1.92	0.34	+	-
At4g25490	C-repeat/DRE binding factor 1 (CBF1/DREB1B)	1.68	0.18	+	+
At4g25470	C-repeat/DRE binding factor 2 (CBF2/DREB1C)	1.56	0.34	+	+
At1g74930	AP2/ERF transcription factor, putative(DREB subfamily)	1.66	0.28	+	-
At1g21910	AP2/ERF domain-containing transcription factor(DREB subfamily)	1.64	0.21	+	-
At4g34410	AP2/ERF domain transcription factor, putative(ERF subfamily)	1.54	0.4	+	+
At5g61600	AP2/ERF domain transcription factor, putative/ethylene responsive element binding factor 8(ERF subfamily)	1.62	0.21	+	-
At1g04250	auxin-responsive protein IAA17/AXR3	1.53	0.43	+	-
At2g33310	auxin-responsive protein IAA13	1.52	0.33	+	-
At1g04240	auxin-responsive protein IAA3/SHY2	1.73	0.69	+	+
At3g25710	bHLH protein family(BHLH32)	1.55	0.21	+	+
At3g60390	homeobox-leucine zipper protein HAT3	1.75	0.61	+	+
At2g46830	MYB-related transcription factor (CCA1)	1.73	0.38	+	+
At5g17300	myb-related family transcription factor	1.71	0.29	-	+
At1g74840	myb-related family transcription factor(CCA1-like)	1.61	0.25	+	-
At1g10170	zinc finger (NF-X1 type) family protein	1.57	0.41	+	+
At5g54470	zinc finger (B-box type) family protein	1.62	0.55	-	-
At3g02380	Zinc finger protein CONSTANS-LIKE 2 (COL2)	1.6	0.42	+	-
At1g66500	zinc finger (C2H2-type) family protein	1.55	0.25	-	-
At1g04500	zinc finger CONSTANS-related similar to Zinc finger protein constans-like 15 (SP:Q9FHH8)	1.55	0.49	+	-
At3g56790	RNA splicing factor-related contains weak similarity to U4/U6-associated RNA splicing factor [Homo sapiens] gi|2853287|gb|AAC09069	1.65	0.43	+	-
At5g01270	Ser-5 specific C-terminal domain phosphatase(CPL2)	1.64	0.27	+	+
Unclassified					
At1g34355	forkhead-associated domain-containing protein / FHA domain-containing protein	1.73	0.41	-	+

At5g43620	S-locus protein 4-related	1.57	0.3	+	-
At3g61620	exonuclease RRP41	1.54	0.34	+	-
At3g60260	phagocytosis and cell motility protein ELMO1-related	1.54	0.36	-	+
At1g11460	nodulin MtN21 family protein	1.62	0.53	+	+
At3g43660	nodulin - like protein	1.57	0.29	+	-
At1g21140	nodulin, putative similar to nodulin 21 (N-21) [Glycine max] SWISS-PROT:P16313	1.55	0.3	+	+
At3g43630	nodulin -related protein	1.53	0.22	+	+
At5g48850	male sterility MS5 family	1.52	0.25	+	-
At1g67880	N-acetylglucosaminyltransferase - related	1.51	0.28	-	+
At5g65630	bromodomain-containing protein	1.5	0.24	+	+
At3g07360	armadillo/beta-catenin repeat family protein / U-box domain-containing protein contains Pfam domain, PF00514: Armadillo/beta-catenin-like repeats and Pfam, PF04564: U-box domain	1.5	0.14	+	+
At1g69840	band 7 family protein strong similarity to hypersensitive-induced response protein [Zea mays] GI:7716466; contains Pfam profile PF01145: SPFH domain / Band 7 family	1.5	0.24	+	+
At2g03750	sulfotransferase family	1.63	0.54	+	-
At1g09800	tRNA pseudouridine synthase family protein, contains Pfam profile PF01416: tRNA pseudouridine synthaseß	1.65	0.42	+	+
Unknown					
At5g61820	expressed protein MtN19, Medicago truncatula, EMBL:MTY15367	1.54	0.25	+	-
At2g37880	expressed protein contains Pfam profile PF04759: Protein of unknown function, DUF617	2.18	0.85	+	-
At1g73510	hypothetical protein	1.91	1.1	-	+
At5g17460	expressed protein	1.75	0.4	+	-
At5g41590	hypothetical protein	1.72	0.41	+	-
At3g12320	expressed protein	1.71	0.46	+	+
At1g72240	expressed protein	1.71	0.42	+	-
At3g22090	hypothetical protein	1.7	0.36	-	-
At2g21640	expressed protein	1.7	0.5	+	+
At5g06980	expressed protein	1.64	0.34	-	+
At4g22370	hypothetical protein	1.6	0.3	+	+
At1g62935	hypothetical protein	1.6	0.42	-	-
At1g48405	hypothetical protein	1.58	0.43	+	+
At5g02680	hypothetical protein	1.55	0.39	+	-
At1g73210	expressed protein	1.52	0.4	-	-
At1g05360	Unknown protein	1.51	0.35	+	-
At5g35110	hypothetical protein	1.5	0.43	+	+
At5g62170	Unknown protein	1.6	0.2	-	+

Table 5-5. Genes that are negatively regulated by 150 mM NaCl in *wrky33-1* mutant.

AGI	Putative identity	F.C.	S.D.	W-box	W-box-like
Cell growth and division					
At1g03870	fasciclin-like arabinogalactan-protein (FLA9)	0.65	0.19	+	-
At3g21070	ATP-NAD kinase family protein contains Pfam domain, PF01513: ATP-NAD kinase	0.62	0.11	+	+
At3g23450	glycine-rich protein	0.62	0.21	-	+
At2g05510	glycine-rich protein	0.66	0.08	+	-
At2g05520	glycine-rich protein (GRP)	0.52	0.17	+	+
At5g39190	germin-like protein (AtGER2)	0.48	0.14	+	+
At1g18980	germin-like protein, putative	0.58	0.1	+	-
At5g39110	germin-like protein (GLP6)	0.64	0.13	+	+
At5g38940	germin-like protein, putative	0.59	0.09	+	-
Cell rescue, defense, death and aging					
At1g74590	glutathione S-transferase, putative(AtGSTU10)	0.5	0.14	+	-
At1g69920	glutathione S-transferase, putative(AtGSTU12)	0.59	0.08	+	-
At1g69930	glutathione S-transferase, putative(AtGSTU11)	0.62	0.15	+	+
At1g55020	lipoxygenase (LOX1)	0.67	0.1	+	+
At5g05340	peroxidase, putative(PER52)	0.36	0.32	+	+
At4g08770	peroxidase, putative(PER37)	0.52	0.06	-	+
At4g08780	peroxidase, putative(PER38)	0.58	0.1	+	+
At5g06730	peroxidase, putative(PER54)	0.63	0.17	+	-
At4g37520	peroxidase, putative (PER50)	0.61	0.07	+	+
At1g68850	peroxidase, putative ATP23a(PER11)	0.46	0.13	+	+
At1g14550	anionic peroxidase, putative(PER5)	0.45	0.07	+	+
At2g43510	trypsin inhibitor -related	0.62	0.17	+	-
At2g15220	secretory protein, putative similar to NtPRp27	0.55	0.06	+	+
At4g11650	osmotin-like protein (OSM34)	0.62	0.16	+	+
At5g43570	serine protease inhibitor, potato inhibitor I-type family protein	0.62	0.11	+	+
At3g04720	hevein-related protein precursor (PR-4)	0.57	0.18	+	+
At1g77120	alcohol dehydrogenase (ADH)	0.57	0.09	-	-
At5g24760	alcohol dehydrogenase (ADH), putative	0.66	0.11	+	-
At1g19610	plant defensin protein, putative (PDF1.4)	0.63	0.18	+	+
At5g57625	pathogenesis-related protein PR1 precurosr/ allergen V5/Tpx-1-related family protein	0.52	0.26	+	+
At3g14225	GDSL-motif lipase/hydrolase protein(GLIP4)	0.35	0.29	+	+
At5g40990	GDSL-motif lipase/hydrolase protein(GLIP1)	0.6	0.03	-	+
At2g38870	protease inhibitor -related	0.67	0.16	-	+
Cellular communication/Signal transduction					
At3g51920	calmodulin 9	0.53	0.01	+	+

At4g33050	calmodulin-binding family protein/EDA39 (embryo sac development arrest 39)	0.64	0.17	+	+
At4g23170	protein kinase family protein, similar to receptor-like protein kinase 5	0.38	0.07	+	-
At3g46270	receptor protein kinase-related	0.44	0.08	+	+
At3g55150	exocyst subunit EXO70 family	0.56	0.15	+	+
At2g39380	exocyst subunit EXO70 family	0.44	0.07	+	-
At3g46280	protein kinase-related contains similarity to light repressible receptor protein kinase. gi|1321686|emb|CAA66376	0.65	0.03	+	+
At5g15080	serine/threonine specific protein kinase - related	0.67	0.18	+	-
At2g40180	protein phosphatase 2C (PP2C), putative	0.61	0.06	+	-
At2g19190	light repressible receptor protein kinase, putative	0.37	0.28	+	+
At4g11530	serine/threonine kinase-related protein (fragment)	0.65	0.12	+	-
At4g04960	receptor lectin kinase, putative	0.66	0.18	+	+
At5g06740	lectin-related protein kinase	0.66	0.14	+	+
Cellular structure, organization and biogenesis					
At5g24540	glycosyl hydrolase family 1 protein	0.37	0.29	+	+
At5g24550	glycosyl hydrolase family 1 protein	0.4	0.28	+	-
At3g60120	glycosyl hydrolase family 1 protein	0.46	0.07	+	-
At4g16260	glycosyl hydrolase family 17(beta-1,3-glucanase class I precursor)	0.55	0.22	+	+
At5g24090	glycosyl hydrolase family 18 (acidic endochitinase)	0.55	0.11	+	-
At1g67980	S-adenosyl-L-methionine:trans-caffeoyl-Coenzyme A 3-O-methyltransferase homologue mRNA, complete cds	0.65	0.1	+	+
At4g34050	caffeoyl-CoA 3-O-methyltransferase	0.66	0.11	+	-
Cellular transport and transport facilitation					
At1g15520	ABC transporter family protein	0.62	0.12	-	-
At2g46430	cyclic nucleotide-regulated ion channel (CNGC3)	0.64	0.04	+	-
At1g44100	amino acid permease 5 (AAP5)	0.55	0.11	-	+
At1g77380	amino acid carrier, putative	0.62	0.14	+	-
At3g13610	oxidoreductase, 2OG-Fe(II) oxygenase family	0.51	0.15	+	-
At4g03540	hypothetical integral membrane protein common family	0.62	0.12	+	+
At2g35730	heavy-metal-associated domain-containing protein	0.61	0.06	+	+
At4g12550	protease inhibitor/seed storage/lipid transfer protein (LTP) family	0.42	0.14	+	-
At1g05450	protease inhibitor/seed storage/lipid transfer protein (LTP)-related similar to geranyl diphosphate synthase large subunit [Mentha x piperita] GI:6449052	0.65	0.16	+	+
At3g22600	protease inhibitor/seed storage/lipid transfer	0.63	0.11	+	-

	protein (LTP) family				
At2g44790	uclacyanin II	0.59	0.13	+	-
At2g02850	plastocyanin-like domain containing protein (plantacyanin, putative)	0.64	0.05	+	+
At2g30750	cytochrome p450 71A12	0.35	0.31	-	+
At4g31950	cytochrome P450 family	0.55	0.08	+	+
At2g45970	cytochrome P450, putative	0.66	0.12	+	+
At3g43890	CHP-rich zinc finger protein, putative	0.6	0.12	+	-
At2g34420	photosystem II type I chlorophyll a /b binding protein	0.53	0.19	-	-
At1g26400	FAD-linked oxidoreductase family	0.45	0.26	-	+
At1g26420	FAD-linked oxidoreductase family	0.53	0.23	+	+
At2g46750	FAD-linked oxidoreductase family	0.67	0.16	+	-
At1g54000	myrosinase-associated protein, putative	0.67	0.12	+	-
At4g32490	plastocyanin-like domain containing protein	0.67	0.06	+	-
Metabolism					
At4g25900	aldose 1-epimerase family	0.67	0.06	+	-
At2g02010	glutamate decarboxylase	0.55	0.09	+	+
At2g39400	hydrolase, alpha/beta fold family	0.56	0.18	+	+
At4g36610	hydrolase, alpha/beta fold family	0.63	0.2	+	-
At3g55190	esterase/lipase/thioesterase family	0.61	0.09	+	-
At4g26270	pyrophosphate-dependent phosphofructo-1-kinase	0.61	0.19	+	+
At5g14590	isocitrate dehydrogenase [NADP+], putative	0.54	0.15	+	-
At2g30140	glycosyltransferase family	0.66	0.15	+	-
At5g55120	similar to VTC2 (VITAMIN C DEFECTIVE 2)	0.48	0.04	+	+
At2g26560	lipid acyl hydrolase(PLP2)	0.51	0.09	+	+
At3g01420	feebly-related protein	0.67	0.16	+	-
Protein fate (folding, modification, and destination)					
At5g07460	peptide methionine sulfoxide reductase-related protein	0.63	0.04	+	+
At3g57810	OTU-like cysteine protease family protein	0.56	0.07	+	-
At1g53980	polyubiquitin -related	0.64	0.14	+	+
Transcription					
At3g12910	transcription factor; similar to ANAC042	0.36	0.09	+	+
At5g40590	CHP-rich zinc finger protein, putative	0.47	0.19	+	+
At4g18170	WRKY family transcription factor 28	0.59	0.08	+	+
At5g60710	zinc finger (C3HC4-type RING finger) protein family	0.66	0.08	+	+
At3g53820	zinc finger (C2H2 type) protein family	0.64	0.13	+	+
At1g56380	mitochondrial transcription termination factor family protein	0.45	0.17	+	+
Unclassified					+
At2g27550	centroradialis protein, putative (CEN) strong similarity to SP|Q41261 CENTRORADIALIS protein	0.62	0.16	+	+
At5g52390	photoassimilate-responsive protein PAR-related protein	0.47	0.23	+	+
At5g09480	proline-rich protein family	0.52	0.09	+	-

At1g53140	dynamin family protein low similarity to dynamin-like protein E	0.53	0.08	+	-
At5g53830	VQ motif-containing protein contains PF05678: VQ motif	0.57	0.12	-	-
At3g02550	lateral organ boundaries (LOB) domain protein 41 (LBD41)	0.58	0.15	+	-
At4g38080	proline-rich protein family	0.64	0.15	+	+
At5g41040	transferase family	0.66	0.09	+	-
At3g47380	invertase/pectin methylesterase inhibitor family protein	0.67	0.07	+	+
At2g33780	VQ motif-containing protein contains PF05678: VQ motif	0.45	0.1	+	-
Unknown					
At1g56320	expressed protein	0.56	0.23	+	+
At1g65500	expressed protein	0.56	0.04	+	+
At4g24130	expressed protein contains Pfam profile PF04398: Protein of unknown function, DUF538	0.58	0.16	+	+
At5g03390	expressed protein contains Pfam profile PF03478: Protein of unknown function (DUF295)	0.61	0.05	+	-
At1g28190	expressed protein	0.62	0.06	+	-
At5g59790	expressed protein	0.62	0.18	+	-
At5g12170	expressed protein similar to unknown protein (pir||T09909)	0.62	0.08	-	+
At5g38310	hypothetical protein	0.62	0.09	+	+
At1g52550	expressed protein	0.63	0.07	-	-
At5g57000	expressed protein similar to unknown protein (gb|AAF21159.1)	0.65	0.07	+	-
At3g55600	expressed protein	0.65	0.1	+	+
At5g22270	expressed protein similar to unknown protein (gb AAF02129.1)	0.52	0.29	+	+
At1g53970	hypothetical protein	0.36	0.05	+	+
At3g31390	hypothetical protein	0.54	0.06	+	-
At5g44990	hypothetical protein	0.64	0.12	+	+
At5g53820	expressed protein similar to ABA-inducible protein [Fagus sylvatica] GI:3901016, cold-induced protein kin1 [Brassica napus] GI:167146	0.67	0.12	+	-
At4g34120	CBS domain containing protein	0.67	0.05	+	+

promoter region contains three standard W-box *cis*-elements (data not shown), indicating *WRKY28* could be a target of WRKY33. Researches on WRKY53 and WRKY70 revealed that one WRKY gene can regulate the activity of several other WRKY genes (Miao *et al.*, 2004; Li *et al.*, 2004).

To validate the changes of some genes, we used qRT-PCR to examine the

changes of four differential genes in the *wrky25-1* dataset and, nine genes including five down-regulated genes and four up-regulated genes, for the *wrky33-1* dataset. These expression profile data obtained by microarray analysis corresponded well with those obtained by qRT-PCR analysis of (Fig. 5-11A, B).

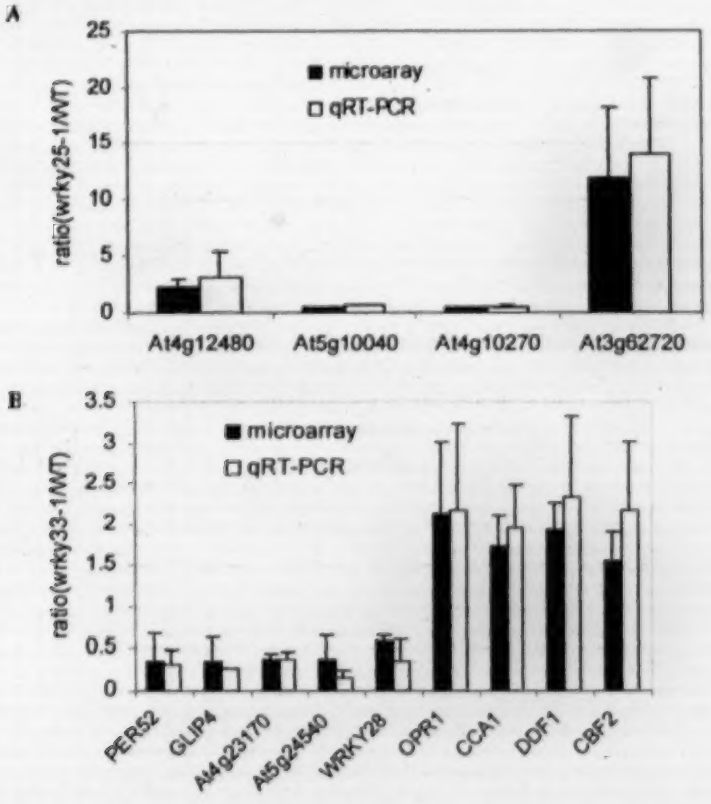


Fig. 5-11. qRT-PCR validation of selected differentially expressed genes between *wrky25-1*(A) or *wrky33-1* (B) mutant and WT under saline condition. Data were the mean values of three biological replicates. Error bars represent S.D.

Only three genes, At2g33780 (VQ motif-containing protein), At2g36800 (glucosyl transferase -related), At3g02550 (lateral organ boundaries (LOB) domain protein 41 (LBD41) were commonly regulated in both *wrky25* and *wrky33*, indicating the WRKY25 and WRKY33 TFs may regulate the activity of quite different downstream genes.

W boxes are highly enriched in WRKY25 and WRKY33 regulon promoters

The regulatory effect of WRKY TFs is primarily through their binding with conserved W box elements on the promoters of specific genes (Ulker and Somssich, 2004). The coregulation of different genes may reflect transcriptional control mechanisms shared and may be based on conserved promoter elements. We inspected 1 kb upstream of the predicted translation start site (ATG). We found the binding site for WRKY transcription factors (W boxes; (C/T)TGAC(T/C); Eulgem *et al.*, 2000; Ulker and Somssich, 2004) was highly enriched in the promoters (Table 5-6). WRKY proteins bind to sequences with an invariant TGAC core, which is in most cases preceded and followed by a T or C. This (C/T)TGAC(T/C) hexamer appears 464 times on both strands in the 208 regulon promoters of WRKY33 regulon, an average of 2.2 copies per promoter (Table 5-6). In WRKY25 regulon, this (C/T)TGAC(T/C) hexamer appears 53 times on both strands in the 31 regulon promoters, an average of 1.7 copies per promoter (Table 5-6).

In contrast, using the most stringent definition for WRKY binding sites (a TTGACC/T hexamer), we still detected 162 genes (78%) whose promoters contain either a TTGACC or TTGACT motif or both. In total, we found a significant over-representation of 376 potential binding sites, an average of 1.8 copies per promoter, in these 57 *WRKY33*-regulated gene promoters (Table 5-6). We note that the *P* value for distribution of W-box hexamers is also significant. 46 of 208 *WRKY33* regulon promoters do not contain a copy of the TTGACC/T motif. However, we also observed that 116 promoters contain the W-box-like hexamer TTGACA. Previous report showed that this W-box-like motif is found 31 times within the 26 *PR-1* regulon promoters (Maleck *et al.*, 2000), which is twice the statistically expected frequency. Clustered occurrences of W boxes are common among promoters of WRKY-regulated genes. Association of these *cis*elements as palindromes or direct repeats with spacing of less than 20 bp results in binding cooperativity and synergistic transcriptional activation (Rushton *et al.*, 1996; Fukuda 1997; Eulgem *et al.*, 1999). We found both the core and stringent W-box elements (TTGAC and TTGACC/T) arranged in a palindromic or directly repeated manner on many promoters from the *WRKY33* regulon.

Table 5-6. Occurrence of W-box and W-box-like motifs in the *WRKY25* and *WRKY33* regulons.

oligomer	Absolute number of this oligomer in query set	Number of sequences in query set containing oligomer	average frequency	p-value from binomial distribution	data set
WRKY33 regulon					
TTGACT	169	90/121	1.9	9.09E-08	down
	77	47/87	1.6	7.39E-02	up
TTGACC	92	59/121	1.6	7.81E-04	down
	38	32/87	1.2	8.53E-02	up
CTGACC	20	20/121	1.0	7.49E-02	down
	20	17/87	1.2	4.22E-02	up
CTGACT	26	24/121	1.1	9.03E-02	down
	22	20/87	1.1	8.18E-02	up
TTGACA(W-box-like)	110	70/121	1.6	3.34E-02	down
	68	46/87	1.5	8.47E-02	up
GTGACT	39	36/121	1.1	5.90E-02	down
	21	16/87	1.3	2.14E-02	up
TTGACG	43	35/121	1.23	5.42E-02	down
	38	29/87	1.31	2.31E-02	up
WRKY25 regulon					
TTGACT	15	10/16.	1.5	1.33E-01	down
	18	9/15.	2.0	0.163	up
TTGACC	7	6/16.	1.2	1.99E-01	down
	6	5/15.	1.2	2.11E-01	up
CTGACT	3	3/16.	1.0	2.46E-01	down
	4	4/15.	1.0	1.89E-01	up
TTGACA(W-box-like)	19	11/16.	1.7	3.34E-02	down
	19	11/15.	1.7	8.52E-02	up
GTGACT	5	4/16.	1.3	2.23E-01	down
	6	5/15.	1.2	1.82E-01	up
TTGACG	3	3/16.	1.00	2.03E-01	down
	4	3/15.	1.33	2.22E-01	up

Transcription factors of the TGA subgroup of bZIP proteins (TGA-bZIPs) are positive regulators of INA-induced *PR-1* expression. They recognize a pentameric element, TGACG that potentially overlaps the W box consensus (Schindler *et al.*, 1992). The TGAC W box core, however, is followed by C or T in all available reports of WRKY/W box interactions. Thus, a distinction between binding sites for these two regulatory factor families can be predicted. We observed that 81 TTGACG motifs were found in 64 promoters.

Although WRKY factors show high binding affinity to a DNA sequence designated the W box, (C/T)TGAC(T/C), altered binding preferences have also been observed (Sun *et al.*, 2003). In barley, a WRKY transcription factor, SUSIBA2 implicated in sugar signaling was found to be able to bind the variant W-box motif GTGACT in the *isol* promoter. We counted 60 GTGACT motifs from 52 WRKY33 regulon promoters. We propose that the remaining 13 genes (6%) whose promoters do not contain W-box or W-box-like motifs may be the indirect targets of WRKY33.

As for WRKY25 regulon, we also detected 25 genes (81%) whose promoters contain W-box motif, and 22 genes (71%) contain W-box-like motif in their promoter regions. Furthermore, we found that 24 genes (77%) contain the stringent W-box motif (TTGACT/C) in their promoters. These data suggest that both WRKY25 and WRKY33 can positively and negatively control the expression of a distinct subset of genes, either directly or indirectly.

Discussion

Soil salinity has detrimental effects on plant growth and crop yields in agricultural production. Excess salts in the soil interfere with the plants' ability to take up nutrients and cause water deficit, followed by a decrease of the K^+/Na^+ ratio in cells. To minimize stress damage and to re-establish ion homeostasis, plants respond and adapt to salt stress by changing gene expression patterns, metabolic activity, and ion and water transport (Zhu, 2003). Many genes with various functions are induced by high salinity stress, and their gene products function in stress tolerance (Chinnusamy *et al.*, 2005). Numerous evidences indicate that specificity and cross-talk exist between various abiotic stresses as well as between biotic and abiotic stress (Seki *et al.*, 2003; Zhu *et al.*, 2001, 2002).

Proteins belonging to the WRKY superfamily of transcription factors are known to regulate a multiplicity of biological processes. Members of the "plant-specific" WRKY transcription factor family have been implicated in the regulation of genes involved in pathogen-induced stress (Dong *et al.*, 2003; Eulgem *et al.*, 2000; Eulgem and Somssich, 2007). Some WRKY genes were also found to be responsive to salt, as well as drought, and cold stresses (Seki *et al.*, 2002; Jiang and Deyholos, 2006). They

are also believed to play an important role in leaf senescence (Robatzek and Somssich, 2001; Miao *et al.*, 2004). Recent studies suggest that biotic and abiotic stress responses may share common components, indicating that there is cross-talk between abiotic and biotic stress signal transduction pathways (Park *et al.* 2001; Mengiste *et al.* 2003; Chini *et al.* 2004). This study shows that two members of Arabidopsis *WRKY* gene family, WRKY25 and WRKY33, are involved in modulating abiotic stress responses.

WRKY 25 and WRKY33 are involved in abiotic stress responses

WRKY genes have been duplicated many times during plant evolution, resulting in a large gene family involved in regulating a large set of genes and thereby ensuring proper cellular responses to physiological processes, internal and external stimuli (Zhang and Wang, 2005). It is believed that functional redundancy might exist between members of WRKY gene family (Ulker and Somssich 2004). Therefore, a more practical approach to characterizing them is to study a group of phylogenetically closed WRKY genes together, as demonstrated by Xu *et al.*(2006). According to the phylogenetic tree of the 72 WRKY TFs in *Arabidopsis*, *WRKY2, WRKY20, WRKY25, WRKY26* and *WRKY33* genes belong to group Ia containing two WRKY domains. However, considering that WRKY20 and WRKY26 were not responsive to salt treatment, we studied WRKY25 and WRKY33 together. Our qRT-PCR results showed that the transcript abundance of *WRKY25* and *WRKY33* was increased by multiple abiotic stresses (Fig. 5-6A, B). Although these two genes were similarly induced by NaCl treatment, differences in their responses to other abiotic stresses were detected, for instance, *WRKY25* transcript abundance was increased by cold and heat treatments at 24 h time point (Fig. 5-6A), while *WRKY33* by cold treatment at both 6 and 24 h (Fig. 5-6B). In our experiment, *WRKY33* is not responsive to oxidative stress treatment induced by 10 μM Paraquat (Fig. 5-6B), whereas Zheng *et al.*(2006) reported that *WRKY33* transcript level is increased by 100 μM Paraquat treatment. This difference can be explained by the different concentrations of Paraquat used. Overall the different responses of *WRKY25* and *WRKY33* to abiotic stresses suggest that they may play different roles in Arabidopsis under abiotic stress.

WRKY proteins have been identified as transcription factors that bind DNA sequences containing W-boxes (C/TTGACC/T). The nuclear localization of WRKY25

and WRKY33 using GFP as a reporter gene suggests that they may possess transcription factor activity (Fig 5-5). Among the target genes identified by microarray (Tables 5-2 to 5-5), many contain W box or W box-like motif in their promoters. In order to test the WRKY protein–W-box-like motif interaction, we tried to express WRKY25 and WRKY33 in *E. coli* using pET32a(+) vector and BL21(DE3) or Rosetta gami strains (Novagen), which proved unsuccessful, most probably due to the high frequency of presence of rare codons encoding Arg, Lys and His amino acids (data not shown).

qRT-PCR assay demonstrated that transcripts of both *WRKY25* and *WRKY33* were most abundant in leaves, roots, and also in stems and flowers (Fig. 5-4). These mRNA accumulation patterns were generally consistent with the GUS expression patterns observed in promoter-GUS fusions of each gene (Fig. 5-8). This consistence between mRNA and GUS staining pattern can be partly explained by the fact that we used a co-transformation strategy to avoid the enhancer effect of CaMV35S promoter on the nearby reporter gene expression. Using promoter-GUS fusion, we detected the inducibility of GUS expression by salt for both PWRKY25 and PWRKY33 (Fig. 5-8), which were also induced by JA and drought, respectively (Fig. 5-8). However, we failed to observe the inducibility of PWRKY25 and PWRKY33 by other stress treatments tested, which is different from qRT-PCR results shown in Fig. 5-6. The minor difference on GUS expression between untreated and treated samples may only be detected by performing a quantitative GUS enzymatic assay. In addition, promoter fusion study using GUS may still not be a perfect way of studying gene expression patterns because regulatory elements may also exist within the coding region, introns, or downstream regions instead of the 5' upstream region (Taylor 1997).

ABA is an important stress hormone extensively involved in response to abiotic stresses such as salt, drought and osmotic stress. At least four regulatory systems exist for the regulation of salt- and osmotic stress-inducible genes in *Arabidopsis thaliana* (Shinozaki & Yamaguchi-Shinozaki 1997; Shinozaki *et al.*, 2003). Although most salinity-inducible genes are regulated by an ABA-dependent pathway, some are not dependent on ABA for signalling. A conserved *cis*-acting element, referred to as DRE/CRT, functions in stress-inducible gene expression in an ABA-independent

manner (Shinozaki *et al.*, 2003). In ABA-dependent pathways, the ABRE (ABA-responsive element) is a major *cis* -acting element in stress-responsive gene expression (Shinozaki *et al.*, 2003). Genetic analysis of two *Arabidopsis* ABA-deficit mutants, *aba1/los6* and *aba3/los5*, in which genes encoding molybdenum cofactor sulfurase (MCSU) and zeaxanthin epoxidase (ZEP) are dysfunctional, showed that ABA is necessary for the salt-stress induced expression of some late-embryogenesis-abundant (LEA) genes (Xiong *et al.*, 2001, 2002a, 2002b). qRT-PCR analysis of Arabidopsis roots treated by 50 μM ABA for 6 h demonstreated that the transcript abundance of *WRKY25* and *WRKY33* was slightly increased compared to the control. The putative promoter region of *WRKY25* does not contain any ABRE element, whereas *WRKY33* contains one single ABRE element (Table 5-1). Skriver et al (1991) reported that polymerized copies of ABREs can confer ABA responsiveness to a minimal promoter, whereas a single copy of ABRE is not sufficient for the full ABA response. Our results showed that salt-induced *WRKY25* transcript accumulation does not require ABA, while *WRKY33* is partially ABA-dependent, suggesting WRKY25 and WRKY33 may work in divergent pathways with respect to ABA. *wrky25* and *wrky33* mutants did not show significant difference from WT on the ABA medium plates (Fig. 5-9), however, *WRKY25*- and *WRKY33*- overexprsssing plants were more sensitive to ABA treatment (Fig. 5-10), suggesting that WRKY25 and WRKY33 might work as positive regulators in mediating plant responses to ABA. Previous researches with rice *WRKY* genes have revealed positive and negative regulators of ABA signaling (Xie *et al.*, 2005).

Arabidopsis sos mutants were identified by genetic screening of seedlings that were hypersensitive to NaCl stress, characterized by retarded root growth (Liu & Zhu 1998; Liu *et al.* 2000; Shi *et al.* 2000). In particular, the *sos1*, *sos2*, and *sos3* mutants are specifically hypersensitive to Na^+ and Li^+ ions. Genetic and physiological data indicate that SOS1, SOS2, and SOS3 function within the same pathway, in which the SOS3–SOS2 complex regulates the expression level of *SOS1* and activates the transport activity of SOS1, which leads to Na^+ tolerance (Qiu *et al.* 2002; Zhu 2003). In addition, salt stress responses were also shown to be mediated by SOS-independent signaling pathways with the identification of several protein kinases activated by salt or osmotic stress (Mikolajczyk *et al.*, 2000; Zhu 2001). In *Arabidopsis* seedlings, a 40-

kDa kinase similar to ASK1 (serine/threonine kinase 1) was identified as being rapidly activated by hyperosmotic stress in a calcium- and ABA-independent manner. Osmotic stress activation of the *Arabidopsis* 40-kDa protein kinase is not impaired by the *sos3* mutation, implying that its activation is independent of the SOS pathway (Hoyos and Zhang, 2000). Our results showed that salt-induced *WRKY25* or *WRKY33* transcript accumulation was not influenced in any of the three *sos* mutants, indicating both WRKY25 and WRKY33-mediated salt response is independent of the SOS signaling pathway.

Knock-out mutation by T-DNA insertion and overexpression are two of the most frequently used techniques to characterize gene function. We found that *wrky25* mutant did not show any significant difference under stress condition compared to WT control, while *wrky33* is slightly more sensitive to salt treatment (Fig. 5-9). The observation suggests that functional redundancy exists between WRKY TFs within group Ia. Accordingly, the double mutant *wrky25wrky33* is more sensitive to salt treatment, although its sensitivity to other types of abiotic stress still needs to be tested. In the case of Arabidopsis WRKY proteins, structurally related WRKY18, WRKY40, and WRKY60 have been shown to have functional redundancy (Xu *et al.* 2006). On the other hand, AtWRKY44 and AtWRKY10 may act much less extent in functional redundancy (Johnson *et al.* 2002; Luo *et al.* 2005).

Overexpression of TFs offers an alternative and complementary strategy to knockout analysis as it is less affected by functional redundancy. In plants, there are several examples in which knockouts of TF genes failed to generate informative phenotypes, but overexpression of these TFs revealed gene functions (Baima *et al.*, 2001; van der Graaff *et al.*, 2002; Zhang 2003). Overexpression of *WRKY25* and *WRKY33* using the constitutive CaMV35S promoter demonstrated similar phenotypies, i.e. enhanced tolerance to salt stress, but more sensitive to oxidative and ABA treatments (Fig. 5-10). Our result on the sensitivity of *WRKY25*-overexpressing plants to oxidative stress is consistent with a previous report (Rizhsky *et al.*, 2004). These data suggest that both WRKY25 and WRKY33 contribute to salt tolerance in Arabidopsis.

WRKY25 and WRKY33 regulate salt stress response by targeting to diverse downstream genes

The role of WRKY25 and WRKY33 in salt stress response was further dissected by identifying their target genes through full-genome microarrays. The number of differentially expressed genes identified between *wrky25* and WT plants is 31, while the number increased to be 208 between *wrky25* and WT plants under a moderate salt treatment (Tables 5-2 to 5-5). The numbers of differentially expressed genes identified reflect the consequence of disrupting *WRKY25* and *WRKY33* expression, which is consistent with the mutant phenotypes under salt stress condition (Fig. 5-9). There are only three common genes between WRKY25 and WRKY33 regulons, suggesting that WRKY25 and WRKY33 have separate roles in salt stress response in addition to common ones. The identified target genes of WRKY33 were classified into ten categories, with many genes implicated in cell defense, cellular transport, signal transduction, metabolism and transcription, and still the identiies of many genes are not clear yet (Tables 5-4 and 5-5), indicating a diverse roles of these target genes regulated directly or indirectly by WRKY33. We note that among the differentially expressed genes identified, many are those encoding transcription factors, especially in the WRKY33 regulon (Tables 5-4 and 5-5), which suggests that WRKY33 can relay the salt stress signal to downstream TFs by controlling their expression.

Inspection of 1 kb upstream regions of these target genes demonstrated that W boxes are highly enriched in WRKY25 and WRKY33 regulon promoters (Table 5-6); however, it is also possible that WRKY25 and WRKY33 interact with other factors directly or indirectly to regulate downstream gene expression. The flanking nucleotides around W boxes may affect the binding affinity of WRKY25 and/or WRKY33 transcription factors to their respective target sequences and can serve to discriminate among closely related factors.

Earlier research has demonstrated that the *Arabidopsis* MAPK kinase 2 (MKK2) with its upstream activator MAPK kinase kinase MEKK1 and the downstream MAPKs MPK4 and MPK6 mediate cold and salt stress signaling (Teige *et al.*, 2004). Salt stress induces the expression and activity of AtMEKK1 (Ichimura *et al.*, 2000). AtMPK4 is activated by cold, low humidity, osmotic stress, touch, and wounding (Ichimura *et al.*,

2000). MPK4 can phosphorylate WRKY25 and WRKY33 *in vitro*, and a substrate of MPK4, MKS1 can interact with WRKY25 and WRKY33 (Andreasson *et al.*, 2005), indicating that a MAPK cascade regulates WRKY25 and WRKY33 activity. A previous study with an Arabidopsis MAPK phosphatase 1 mutant (*mkp1*) demonstrated the complexity of the AtMPK4 signalling cascade. *mkp1* is resistant to salinity but hypersensitive to UV-C. A yeast two-hybrid screen showed that MKP1 could interact with AtMPK4 (Ulm *et al.*, 2002). Microarray analysis of *mkp1* indicated that the transcript level of a putative Na^+/H^+-antiporter (CHX17, At4g23700) is negatively regulated by AtMKP1 (Ulm *et al.*, 2002). More recenty research on MEKK1 and MPK4 suggests that their functons are not limited to a single, linear signaling pathway

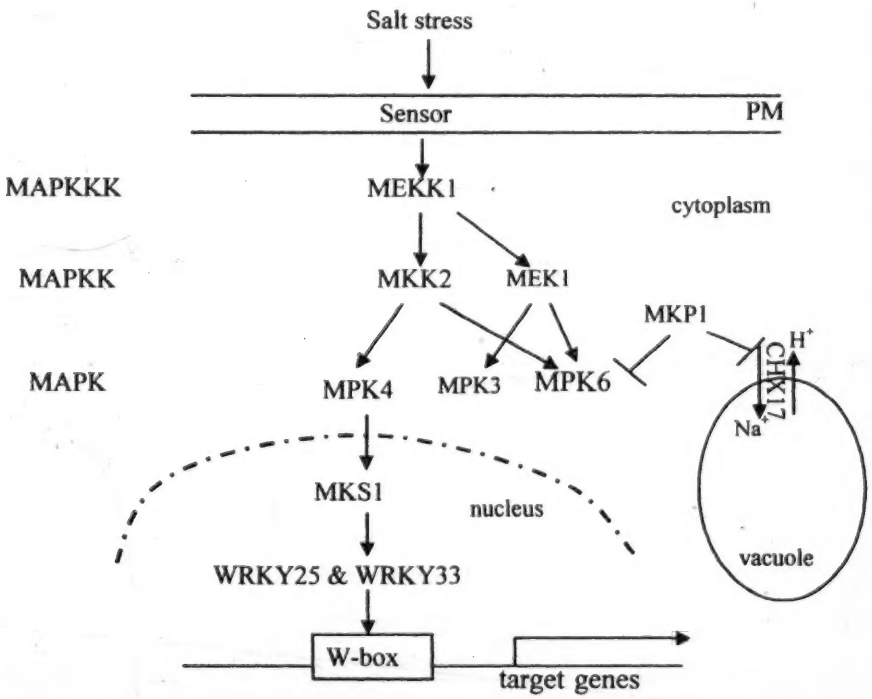


Fig. 5-12. Proposed working model explaining role of WRKY25 and WRKY33 in salt stress responsive pathway. High concentration of salt in the apoplast in sensed by an unknown sensor on the plasma membrane. This is transduced by a MAPK-mediated pathway to MPK4, which can phosphate MKS1. MKS1 activates WRKY25 and WRKY33, which can recognize and bind to the W-box in the downstream target genes and activated their transcription. Arrows indicate positive regulation, and open blocks indicate negative regulation.

pathway (Su *et al.*, 2007), which suggests the existence of other unkown component(s). These results, together with our analysis, suggest that two WRKY transcription factors function in the MPK4-mediated signaling pathways in plant responses to salt stress. Thus, these two transcription factors play a role in responses to both biotic as well as abiotic stresses and, biotic and abiotic stress signals converge at the level of MAPK cascades. On the basis of the results presented here as well as those reported previously (Ulm *et al.*, 2002; Teige *et al.*, 2004; Andreasson *et al.*, 2005), we present a model describing the interrelationships among MAPK signaling components, WRKY25 and WRKY33 in the salt signal transduction pathway (Fig. 5-12). This model, together with other recent studies of the role of MPK4 in salt and ROS signalings (Nakagami *et al.*, 2006; Su *et al.*, 2007), allows a better understanding of salt signal transduction pathway from sensing to the response in plants.

In summary, this study shows that WRKY25 and WRKY33 play important roles in abiotic tress responses. This strengthens our understanding of transcriptional regulation during salt and other types of abiotic stress responses in higher plants and provides a base for future work in this area.

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Chapter 6: General discussion and conclusions

Plant growth and productivity are adversely affected by a variety of abiotic stresses, such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. Plants are also challenged by pathogens including bacteria, fungi, and viruses as well as herbivores. All these stress factors are a menace for plants and prevent them from reaching their full genetic potential and limit the crop productivity worldwide. Abiotic stress is the major cause of crop failure worldwide, and it causes losses worth hundreds of millions of dollars each year. Because of increases in global population, food productivity also needs to be increased. Therefore, it is important to develop stress tolerant crops.

In response to abiotic stress conditions, expression of various genes in plants is up-regulated, which can mitigate the effect of stress and lead to adjustment of the cellular milieu and plant tolerance (Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong and Zhu 2001). During the past two decades, great progress has been achieved in the study of abiotic stress, including identification of key components and the associated signaling pathways through a combination of forward and reverse genetic strategies (Xiong et al., 2002b; Yamaguchi-Shinozaki and Shinozaki K, 2006). In nature, stress does not generally come in isolation and many different stresses may challenge plants at the same time. Hence, there exists cross talk between abiotic stresses (Xiong *et al.*, 2002; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu 2002; Yamaguchi-Shinozaki and Shinozaki, 2006).

Among the abiotic stresses, soil salinity is one of the dominant factors limiting the harvestable yields of crop plants. Secondary salinization caused by poor irrigation water and subsequent drainage is damaging productive agricultural land worldwide. High salinity causes both hyperionic and hyperosmotic stress and can lead to plant demise. High salt depositions in the soil generate a low water potential zone in the soil making it increasingly difficult for the plant to acquire both water as well as nutrients. Therefore, salt stress essentially results in a water deficit condition in the plant and this is similar to drought. Hence, it is essential to understand the control of ion homeostasis and osmotic regulation, and to use this knowledge to engineer crop plants with

enhanced salt tolerance. Arabidopsis is a small model flowering plant that has many advantages. Research on salt toxicity and tolerance using Arabidopsis has produced significant results, and it is now widely held that NHX1 on the vacuolar membrane and SOS1 on the plasma membrane play an important role in decreasing the cytosolic sodium concentration (Apse and Blumwald, 2002; Zhu 2002), which is the basic mechanism for developing salt-tolerant plants. However, much is still unknown about the elusive salt-stress sensor and regulators of ion transporters.

Conventional methods of breeding salt-tolerant crops are rather slow and have had only limited success (Wang *et al.*, 2003), partly due to the fact that plant responses to salt stress are complex and the underling mechanisms remain unclear. Developing salt-tolerant crops through genetic engineering, i.e. introducing genes from one plant to another or overexpressing them in plants has demonstrated exciting success (Bartels and Sunkar, 2005; Yamaguchi and Blumwald, 2005; Zhang *et al.*, 2005). To identify novel salt-responsive genes and proteins will not only contribute to understanding salt signaling mechanisms but also provide new target genes for engineering stress-tolerant plants.

Microarray is a widely used technology to identify stress-responsive genes in many plants (Kawasaki *et al.*, 2001; Seki *et al.*, 2002a, 2002b, Kreps *et al.*, 2002; Rabbani *et al.*, 2003; Wang *et al.*, 2003; Chao *et al.*, 2005; Ma *et al.*, 2006). With the completion of sequencing of genomes of Arabidopsis and rice, microarrays including almost the whole sets of genes encoded by Arabidopsis and rice genomes have been developed and widely used to identify stress-responsive genes (Chao *et al.*, 2005; Ma *et al.*, 2006). Root is the first organ to sense higher concentration of salt in the soil. Therefore, it is necessary to focus on root for salt stress research. However, there is no report using root as the material to study salt stress response. To identify a comprehensive set of salt-responsive genes, we used the QiagenOperon 26 k oligonucleotide microarray to monitor the transcriptome changes in Arabidopsis roots under the treatment of a moderate NaCl concentration (150 mM) by 6, 24 and 48 h (Jiang and Deyholos, 2006). Statistical analysis of our data enabled us to identify many novel salt-responsive genes and highlight potentially important roles for underappreciated gene families, including: several groups of transporters (e.g. MATE,

LeOPT1-like); signalling molecules (e.g. PERK kinases, MLO-like receptors), carbohydrate active enzymes (e.g. XTH18), transcription factors (e.g. members of ZIM, WRKY, NAC), and other proteins (e.g. 4CL-like, COMT-like, LOB-Class 1).The newly identified salt-responsive genes demonstrated the power of full-genome microarray and may be selected as target genes for developing stress-tolerant crops in the future.

Proteomics based on 2-D gel electrophoresis (2-DGE) is another powerful technology for identifying stress-responsive proteins. Coupled with mass spectrometry, 2-DGE has been applied to identify environmental stress responsive proteins in many plants (Agrawal *et al.*, 2005; Yan *et al.*, 2005 & 2006; Hajheidari *et al.*, 2005, 2007; Lee *et al.*, 2007; Li *et al.*, 2007; Nohzadeh *et al.*, 2007; Plomion *et al.*, 2006; Yang *et al.*, 2007). The type of 2-DGE-based comparative proteomics has yielded interesting new insights into stress responses in plants (Yan *et al.*, 2006; Hajheidari *et al.*, 2007; Nohzadeh *et al.*, 2007; Yang *et al.*, 2007). This is partly explained by the fact that mRNA levels of some genes do not show significant changes under stress condition and therefore fail to be captured by RNA-based techniques such as differential display (DD), suppressive subtractive hybridization (SSH), SAGE (serial analysis of gene expression) and microarray, whereas the changes at the protein levels under stress conditions are above a threshold and thus are detected by the 2-DGE based technology. Besides, mRNA levels are not always correlated with those of corresponding proteins, because of post-transcriptional regulation, such as post-translational modifications (PTMs) including glycosylation and phosphorylation. It has been reported that only moderate correlation exists between changes in the levels of specific mRNAs and their corresponding proteins in studies involving yeast (*Saccharomyces cerevisiae*), animals, or Arabidopsis (Gygi *et al.*, 1999; Tian *et al.*, 2004; Mooney *et al.*, 2006). DNA sequence and mRNA expression profiling provide little information about sub-cellular localization, beyond the identification of putative targeting signal sequences and structural domains. These biological realities motivated us to perform an analysis of NaCl stress responses at the proteome level, and to compare these results to our microarray-based studies of similarly treated Arabidopsis roots. We conducted a comparative proteomic analysis of Arabidopsis roots that had been exposed to 150 mM

NaCl for 6 or 48 h through utilizing two-dimensional electrophoresis coupled to tandem mass spectrometry (Jiang *et al.*, 2007). We successfully identified 86 of the differentially abundant proteins, which are implicated in ROS scavenging, signal transduction, translation, cell wall biosynthesis, protein translation, processing and degradation, and metabolism of energy, amino acids, and hormones. At the resolution of approximately 70 individual genes/proteins, we detected poor statistical correlation of these protein expression data with our previous microarray results, supporting the concept that post-transcriptional regulation plays an important role in stress-responsive gene expression (Jiang *et al.*, 2007). However, more proteins need to be identified to determine the generality of this conclusion under different abiotic stress treatments and measurement techniques.

2-DGE-based proteomic study has its own limitations, i.e. inability to resolve membrane proteins and detect low-abundant proteins (Rose *et al.*, 2004), therefore, complementary strategies at the protein and metabolite levels have been developed and are used to gain more insight into the intricate network of plant response to environmental stimuli. One of these is multidimensional protein identification technology (MudPIT), which was developed to allow automated analyses of peptide mixtures generated from complex protein samples (Link *et al.*, 1999). However, while MudPIT is able to provide a catalogue of all the proteins present in a particular sample, it does not generate useful quantitative information and even reliable qualitative comparisons between samples are still technically challenging and time consuming. A second technique, isotope-coded affinity tag (ICAT) in the LC–MS/MS system has also emerged as a tool for quantitative proteomics (Han *et al.*, 2001). However, the quantitative reproducibility and the number of replicate experiments required for statistical significance are yet to be completely resolved and application of ICAT has not been reported in plants (Rabilloud, 2002).

A common problem with current salt stress study is that many experiments are not designed to expose genes that confer salinity tolerance under natural conditions. Many experiments with NaCl do not include supplemental Ca^{2+}, and even NaCl concentrations as high as 250 or 300 mM have been given in one treatment, with no additional Ca^{2+}. It is widely reported that supplemental Ca^{2+} ameliorates Na^{+} toxicity

symptoms in different plant species (Rengel, 1992). Supplemental Ca^{2+} may provide protection against salinity stress through stabilizing cell wall components, plasma membrane lipids, and proteins (Rengel, 1992; Kinraide, 1998). Supplemental Ca^{2+} in a physiological concentration range (1–10 mM) reduced, or even completely prevented, Na^+-induced K^+ efflux from salinized root and leaf tissues in broad bean (*Vicia faba*) and barley (*Hordeum vulgare*) plants (Shabala, 2000; Shabala *et al.*, 2003). Extracellular Ca^{2+} ameliorates NaCl-induced K^+ loss from Arabidopsis root and leaf cells by controlling plasma membrane K^+-permeable channels (Shabala *et al.*, 2006). In our microarray and 2-DGE experiments, Arabidopsis plants were treated by ½ x MS with or without 150 mM NaCl. The concentration of $CaCl_2$ in ½ x MS solution is 1.5 mM, which is within the range of physiological concentration. However, the issue with our NaCl treatment is that 150 mM NaCl was applied in one hit. Instead, the concentration of NaCl could start with a low concentration of NaCl, i.e. 50 mM, and then gradually increased to be 150 mM within a few days. In this way, more natural salt-responsive genes may be identified.

The discrepancy or low correlation between our microarray and 2-DGE data for some gene/protein was also observed. For instance, the manganese superoxide dismutase (MSD1, At3g10920) was identified to be down-regulated at the transcript level by salt treatment, but it was identified to up-regulated at the protein level (Table 3-1). The nucleoside-diphosphate kinase (NDPK1, At4g09320) was identified to be down-regulated at the transcript level by salt treatment, but it was not found to be repressed at the protein level (Table 3-1). To resolve the discrepancy between microarray and 2-DGE data, Western blotting using specific antibody could be used to validate the 2-DGE data at the protein level. However, this is not very feasible as it is not easy to obtain specific antibodies for these proteins, although some antibodies are commercially available from Agrisera (http://www.agrisera.com/index.php). Alternatively, for those proteins implicated in ROS scavenging such as SOD or APX, enzymatic assays could be preformed to examine their activities in salt-treated roots relative to control.

Three transcription factor genes, *bHLH92*, *WRKY25* and *WRKY33*, whose transcript levels were found to be highly induced by 150 mM NaCl in Arabidopsis

roots, were chosen for further characterization. The bHLH transcription factor gene family is the second largest after MYB in Arabidopsis, and previous studies have shown that members of the bHLH family are involved in diverse physiological processes including cold (Chinnusamy *et al.*, 2003), ABA (Abe *et al.*, 2003), JA (Lorenzo *et al.*, 2004) and more recently, salt stress (Kim and Kim, 2006) signallings. Our study of *bHLH92* demonstrate its abundance was increased by salt, drought, mannitol, JA and cold treatments (Fig. 4-4B), suggesting that *bHLH92* is responsive to multiple abiotic stresses. We also found that the induction of *bHLH92* by NaCl is partly ABA- and SOS2-dependent but independent of SOS1 and SOS3 (Fig. 4-5). Promoter-GUS fusion assay demonstrate that promoter of *bHLH92* is salt, drought and JA inducible (Fig. 4-6). Knock-out mutant of *bHLH92* was sensitive to osmotic stress and slightly to NaCl stress (Fig. 4-8), while constitutive overexpression of *bHLH92* in Arabidopsis increased plants' tolerance to salt, osmotic and oxidative stresses (Fig. 4-9), which indicate that *bHLH92* plays an important role in stress signaling and tolerance. Microarray profiling of *bhlh92* mutant under NaCl treatment identified 19 genes that were differentially expressed between mutant and wild-type, and some genes were known to be involved in abiotic stress or associated with ROS scavenging (Table 4-2). Future study on *bHLH92* can be directed to identify upstream gene(s) regulating bHLH92 activity and its interacting partners by using yeast two-hybrid system or tandem affinity purification (TAP) coupled to LC/MS identification as demonstrated by previous studies (Rohila *et al.*, 2006; Miao and Zentgraf, 2007). We observe that the difference between mutant and WT under stress condition is not very large, and this may suggest a functional redundancy exist between some members of bHLH gene family. Therefore, double, triple or even quadruple knockout mutants for between *bHLH92* and other evolutionally close members of bHLH gene family (Fig. 4-2) could be generated to perform comparative analysis of these composite knockout mutants so that both distinct and overlapping functions of these *bHLH* genes could be revealed. Further work could also involve investigating the transcript levels of abiotic and ABA stress responsive marker genes, such as *RD29A, RD29B, ERD10, KIN1* and *CBF2*, in the *bHLH92*-overexpression lines under NaCl, mannitol or ABA treatment.

WRKY transcription factors have been shown to play an important role in

pathogen responses and leaf senescence (Eulgem *et al.*, 2000; Eulgem and Somssich, 2007). WRKYs form a large gene family with 72 members in Arabidopsis. In a previous microarray profiling assay, 18 WRKY TFs, including *WRKY25* (At2g30250) and *WRKY33* (At2g38470) that were highly induced by 150 mM NaCl treatment in Arabidopsis roots, were identified (Jiang and Deyholos, 2006). Previous cDNA microarray also indentified a few Arabidopsis WRKY genes which are responsive to multiple abiotic stresses (Seki *et al.*, 2002a, 2002b). Recent researches have shown that WRKY25 and WRKY33 play an important role in mediating disease resistance (Zheng *et al.*, 2006, 2007; Lippok *et al.*, 2007), which is proposed to be regulated through a MAPK cascade (Andreasson *et al.*, 2005). We hypothesized that WRKY25 and WRKY33 might also play an important role in abiotic stress response such salt stress tolerance. To test this, we used a combination of reverse genetic methods. We first demonstrate that both *WRKY25* and *WRKY33* were induced by salt, mannitol and cold treatments as well as by other stress treatments (Fig. 5-6). We observed that the inducibility of *WRKY25* by salt stress was ABA- and SOS-signaling pathways independent, whereas the induction of *WRKY33* by salt stress was partially dependent on ABA biosynthesis and signaling but was independent of the SOS pathway (Fig. 5-7). Stable transformed Arabidopsis plants harboring PWRKY25- and PWRKY33-GUS fusion constructs revealed that both promoters were inducible by salt stress as well as by other specific abiotic stresses (Fig. 5-8). Knock-out mutant of *WRKY33* only showed slight sensitivity to salt stress while *WRKY25* knock-out mutants did not exhibit any significant difference from WT under tested stress conditions, suggesting a functional redundancy between evolutionally close WRKY TFs (Fig. 5-2). We further produced *wrky25wrky33* double mutant and found that this double mutant was more sensitive to NaCl treatment than wild-type (Fig. 5-9E & H). Overexpression of *WRKY25* and *WRKY33* separately in Arabidopsis increased plant tolerance to salt but were more sensitive to oxidative and ABA treatments at the seedling stage (Fig. 5-10). These data suggest that *WRKY25* and *WRKY33* are positive regulators of salt stress response while a negative regular of ABA and oxidative stress responses. Finally, full-genome microarray was employed to identify the target genes of WRKY25 and WRKY33 under salt treatment, and 31 and 208 differentially expressed genes between *wrky25* or

wrky33 single mutant and wild-type were identified, respectively (Tables 5-2 to 5-5). Further examination of the 1 kb upstream regions of these differential genes demonstrated that W-box (C/TTGACC/T), which is the *cis*-element recognized and bound by WRKY TFs, is highly enriched in the target gene promoter regions (Table 5-6). Moreover, the different numbers and identity of differential expressed genes identified suggest that WRKY25 and WRKY33 might play a role in salt stress by targeting divergent downstream genes. On the basis of our results as well as those reported previously (Ichimura et al., 2000; Teige *et al.*, 2004; Andreasson *et al.*, 2005), we present a model describing the interrelationships among MAPK signaling components, MEKK1, MKK2, MPK4 as well as WRKY25 and WRKY33 in the salt signal transduction pathway (Fig. 5-12).

Future study can be directed to identify the direct targets of WRKY25 and WRKY33 as well as their interacting partners by using yeast two-hybrid system or tandem affinity purification (TAP) coupled to LC/MS identification as demonstrated by previous studies (Andreasson *et al.*, 2005; Rohila *et al.*, 2006; Miao and Zentgraf, 2007). Further work could also involve investigating the transcript levels of abiotic and ABA stress responsive marker genes, such as *RD29A, RD29B, ERD10, KIN1* and *CBF2*, in the overexpression lines under NaCl treatment. It can also be directed to generate double, triple and quadruple knockout mutants of *WRKY25* or *WRKY33* and other related WRKY genes. Further comparative analysis of these composite knockout mutants should reveal both distinct and overlapping functions of the WRKY genes.

In conclusions, elevated concentration of salt has pleiotropic effects on plant development, morphology and physiology. The changes at both transcript and protein levels in Arabidopsis roots under a moderate salt treatment were monitored using microarray and 2-DGE, respectively. Many differentially expressed genes/proteins were identified, among which many are novel. These help us to better understand plant response to salt stress in the context of regulatory network and data presented in the thesis provide alternative choices for engineering salt-tolerant crops in the future. Characterization of three novel salt-inducible transcription factor genes, *bHLH92*, *WRKY25* and *WRKY33*, using both knock-out and overexpression strategies demonstrate that they play important roles in plant protection against different abiotic

stresses. These findings described in the thesis thus allow a better understanding of the roles of two classes of previously underappreciated TFs, bHLH and WRKY in abiotic stress. The results on WRKY25 and WRKY33 characterization also help to understand the complex interaction and cross-talk between components implicated in biotic and abiotic stresses as they appear to be key regulators connecting biotic stress and abiotic stress signalings.

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